# CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

## III. Active and Passive Anaphylaxis with Synthetic Sugar-Proteins

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In the two preceding papers of this series Avery and Goebel (1, 2) have reported the results of chemo-immunological studies on conjugated carbohydrate-proteins.

In the first of these communications Goebel and Avery (1) described the chemical methods by means of which the p-aminophenol glucosides were synthesized from glucose and galactose and the diazotized substances bound to protein. The two newly synthesized sugar-proteins differ from one another chemically only in specific rotation and molecular configuration. In the second paper, Avery and Goebel (2) reported the antigenic properties and serological specificity of the conjugated carbohydrates. They found that the glucosidic radical of the compound antigen endowed the new complex with specific reactivity. This was demonstrated in two ways. First, when the *same* glucoside is attached to two *different* proteins, as serum globulin or egg albumin, the serum prepared by immunization with either one of the antigens is specifically reactive with the other. Second, when the two *different* glucosides,—glucoside and galactoside—are each combined with the *same* protein, the newly formed compounds are serologically distinct. These facts were found to be true even though the individual glucosides are isomers differing only in the spatial configuration of a single carbon atom.

On the other hand the uncombined glucosides alone were found to be nonantigenic. They failed to induce antibody formation in the animal body and caused no visible precipitation when added to immune sera *in vitro*. However, both glucosides possess the capacity of specifically inhibiting the precipitating action of homologous antisugar-protein serum. When galactoside was mixed with serum prepared by immunization with galacto-globulin,[1] the subsequent addition

[1] The terms employed in this paper to represent the sugar-protein compounds are the same as those used by Avery and Goebel (2).

of galacto-albumin to the mixture did not cause precipitation. Furthermore, when the heterologous glucoside was substituted in the same system, no inhibition of the precipitin reaction occurred. These results demonstrate the specificity of the inhibition phenomenon. Avery and Goebel (2) considered that the uncombined glucosides possess the immunological properties of haptens.

The rôle of carbohydrate in anaphylaxis has been a subject of recent experimental investigation.

Tomcsik (3) working with B. lactis aerogenes, and later Tomcsik and Kurotchkin (4) employing B. lactis aerogenes, the pneumobacillus, and a yeast, isolated carbohydrate substances which produced anaphylactic shock in guinea pigs passively sensitized with homologous immune serum. Lancefield (5) also obtained from streptococci carbohydrate material with which anaphylaxis could be induced in guinea pigs passively sensitized with anti-streptococcus serum. Because of the presence of small amounts of nitrogen in the products, none of these authors felt justified in concluding that the carbohydrate alone was responsible for the shock. Avery and Tillett (6) employing the highly purified polysaccharide of the type-specific pneumococci showed that guinea pigs passively sensitized with homologous anti-pneumococcus rabbit serum were thrown into anaphylactic shock by the subsequent injection of the homologous specific carbohydrate. Guinea pigs could not, however, be actively sensitized with the purified polysaccharides alone. Since the materials used in those experiments were protein-free, and in the case of the Type II and Type III substances also nitrogen free, the results conclusively demonstrate the capacity of complex sugars to induce anaphylactic shock in animals passively sensitized with antibacterial sera.

The immunological specificity of pneumococcus polysaccharides has a close analogue in the serological specificity exhibited by gluco-protein and galacto-protein. The immunologic behavior of the synthesized sugar-proteins led to their use in sensitization experiments. The results, reported in this paper, on anaphylaxis with artificially prepared carbohydrate-proteins confirm and extend the serological findings previously reported. The production of both active and passive anaphylaxis was attempted in order to determine the sensitizing properties of the synthetic antigens and to demonstrate the specificity of the reactions.

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Gluco-globulin represents phenol β-glucoside-azo-globulin.
Gluco-albumin represents phenol β-glucoside-azo-albumin.
Galacto-globulin represents phenol β-galactoside-azo-globulin.
Galacto-albumin represents phenol β-galactoside-azo-albumin.
The globulin was prepared from horse serum, and the albumin from egg white

## EXPERIMENTAL

Guinea pigs weighing 240 to 275 grams were employed. The sensitizing dose, whether serum or sugar-protein, was always injected intraperitoneally. The shocking dose was uniformly injected intravenously into a superficial vein of the hind leg.

For details concerning the chemical procedures involved in the synthesis of the materials, the reader is referred to the article by Goebel and Avery (1).

The serological characteristics of the serum employed and the method of preparation are described by Avery and Goebel (2).

### *Passive Sensitization*

#### *A. Results obtained with sugar-protein compounds*

Eight guinea pigs were injected intraperitoneally with the pooled serum of three rabbits immunized with gluco-globulin. Five guinea pigs each received 5 cc. of serum, one received 3 cc., one received 1 cc., and one 0.5 cc. As previously mentioned, serum of this character possesses the capacity to precipitate complex antigens composed of heterologous protein conjugated with the homologous glucoside. The antigluco-globulin sera used in these experiments had an average titre of specific precipitins, as determined by tests made with gluco-egg-albumin, of 1 to 80,000. By reason of the fact that horse globulin alone when used as antigen does not elicit antibodies reactive with egg-albumin, the high precipitin titre of these sera is obviously dependent upon the conjugated glucoside radical.

Twenty-four hours after the administration of gluco-globulin antiserum, each pig received intravenously 1 cc. of gluco-albumin.

From Table I it can be seen that the five pigs sensitized with 5 cc. of serum all died with typical symptoms of anaphylactic shock.

The animals passively sensitized with 3 cc. and 1 cc., respectively, of antigluco-globulin serum had definite and typical symptoms immediately following the injection of 1 cc. of gluco-albumin but recovered. Pig No. 6, which received 0.5 cc. of serum, exhibited only a slight reaction.

A similar experiment was carried out using antigalacto-globulin serum for sensitization and galacto-albumin as the toxigenic antigen.

Sera obtained from three rabbits immunized with galacto-globulin were pooled; the precipitin titre, as determined with galacto-albumin, averaged 1 to 80,000. Eight guinea pigs were injected intraperitoneally as follows: 5 animals received 5 cc. of serum each, one received 3 cc., one received 1 cc., and one, 0.5 cc. The results given in Table II are equally as definite as those shown in Table I. All

TABLE I

*Passive Anaphylaxis with Anti-Gluco-Globulin Serum*

Reactions induced by the use of the homologous glucoside conjugated with a heterologous protein—Gluco-Albumin

| Guinea pig No. | Anti-gluco-globulin serum i.p. | Interval between injection of serum and shock dose | Shocking dose i.v. | Symptoms | Result |
|---|---|---|---|---|---|
| | *cc.* | *hrs.* | *gluco-egg-albumin* | | |
| 1 | 5 | 24 | 1 cc. | Typical | †3½ minutes |
| 2 | 5 | 24 | 1 cc. | Typical | †3½ minutes |
| 3 | 5 | 24 | 1 cc. | Typical | †2½ minutes |
| 4 | 3 | 24 | 1 cc. | Marked scratching, bucking, coughing, respiratory distress | Definite symptoms followed by recovery |
| 5 | 1 | 24 | 1 cc. | Violent typical symptoms | Definite symptoms followed by recovery |
| 6 | 0.5 | 24 | 1 cc. | Occasional scratching | No reaction |
| | | | *galacto-egg-albumin* | | |
| 7 | 5 | 24 | 1 cc. | None | No reaction |
| | | | *gluco-egg-albumin* | | |
| Same animal 4 hrs. later | — | — | 1 cc. | Typical | †4 minutes |
| | | | *galacto-egg-albumin* | | |
| 8 | 5 | 24 | 1 cc. | None | No reaction |
| | | | *gluco-egg-albumin* | | |
| Same animal 4 hrs. later | — | — | 1 cc. | Typical | †4½ minutes |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

pigs sensitized with homologous immune serum in amounts from 1 to 5 cc. reacted typically and fatally to the intravenous injection of 1 cc. of galacto-albumin; 0.5 cc. of serum was insufficient to sensitize.

TABLE II

*Passive Anaphylaxis with Anti-Galacto-Globulin Serum*

Reactions induced by the use of the homologous galactoside combined with a heterologous protein—Galacto-Albumin

| Guinea pig No. | Anti-galacto-globulin serum i.p. | Interval between injection of serum and shock dose | Shocking dose i.v. | Symptoms | Result |
|---|---|---|---|---|---|
| | *cc.* | *hrs.* | *galacto-egg-albumin* | | |
| 1 | 5 | 24 | 1 cc. | Typical | †3 minutes |
| 2 | 5 | 24 | 1 cc. | Typical | †8 minutes |
| 3 | 5 | 24 | 1 cc. | Typical | †3½ minutes |
| 4 | 3 | 24 | 1 cc. | Typical | †3 minutes |
| 5 | 1 | 24 | 1 cc. | Typical | †3½ minutes |
| 6 | 0.5 | 24 | 1 cc. | Slight scratching and coughing | Very mild reaction with recovery |
| | | | *gluco-egg-albumin* | | |
| 7 | 5 | 24 | 1 cc. | None | No reaction |
| | | | *galacto-egg-albumin* | | |
| Same animal 1 hr. later | — | — | 1 cc. | Typical | †3½ minutes |
| | | | *gluco-egg-albumin* | | |
| 8 | 5 | 24 | 1 cc. | None | No reaction |
| | | | *galacto-egg-albumin* | | |
| Same animal 1 hr. later | — | — | 1 cc. | Typical | †3 minutes |

†Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

In Tables I and II it is also shown that the reactions in guinea pigs induced with sugar-proteins and anti-sera are strictly specific.

Animals No. 7 and No. 8 of Table I received antigluco-globulin serum and 24 hours later were injected intravenously with galacto-albumin. No reaction occurred. Four hours later, the introduction of gluco-albumin induced typical fatal shock. Similarly pigs No. 7 and No. 8 of Table II, sensitized with anti-galacto-globulin serum, were unharmed by gluco-albumin; the subsequent administration of the homologous galacto-albumin antigen caused anaphylactic death of these animals. The animals also serve to demonstrate the fact that gluco-albumin and galacto-albumin are not primarily toxic.

### *B. Results obtained with uncombined glucosides*

The specific inhibitory effect exerted by the glucosides on the precipitin reaction of sugar-protein and anti-sera has been described in detail by Avery and Goebel (2) and has been previously commented upon in this paper. It, therefore, seemed of interest to determine whether anaphylactic shock could be elicited by glucosides alone in passively sensitized guinea pigs, and if not, whether the inhibition which these substances have on the precipitin test would also be evident in the anaphylactic reaction.

As shown in Table III, two pigs (Nos. 1 and 2), sensitized 24 hours previously with antigluco-globulin serum, were injected intravenously with 1 cc. of the uncombined homologous glucoside. No reaction occurred. Two hours later 1 cc. of gluco-albumin injected into the same animal caused prompt anaphylactic death.

In guinea pigs Nos. 3, 4, and 5, the introduction of glucoside alone was followed immediately by an injection of gluco-albumin. Except for slight scratching, no response was elicited.

From these results it may be seen that the injection of glucoside into a sensitized animal exerts a definite but transitory protection against the shocking capacity of material which otherwise would be fatal. That the protective action of the glucoside is specific is demonstrated by guinea pigs Nos. 6, 7, and 8. These animals were injected with the heterologous galactoside; when, immediately thereafter they were given gluco-albumin no protection resulted and they died promptly with typical anaphylactic shock.

Table IV presents the results obtained with guinea pigs, which, after having been sensitized with antigalacto-globulin serum, were protected by the galactoside from the toxigenic effect of galacto-albumin.

TABLE III

*Passive Anaphylaxis with Anti-Gluco-Globulin Serum*

Effect of Uncombined Glucoside

| Guinea Pig No. | Anti-gluco-globulin serum i.p. | Interval between injection of serum and anaphylactic test | Injection of glucoside i.v. | Result | Interval between injection of glucoside and sugar-protein | Injection of sugar-protein i.v. | Symptoms | Result |
|---|---|---|---|---|---|---|---|---|
| | *cc.* | *hrs.* | *glucoside* | | *hrs.* | *gluco-egg-albumin* | | |
| 1 | 5 | 24 | 1 cc. | No reaction | 2 | 1 cc. | Typical | †4 minutes. |
| 2 | 5 | 24 | 1 cc. | No reaction | 2 | 1 cc. | Typical | †4 minutes |
| 3 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Scratches slightly, no other symptoms | No reaction |
| 4 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Scratches slightly, no other symptoms | No reaction |
| 5 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Scratches slightly, no other symptoms | No reaction |
| | | | *galactoside* | | | | | |
| 6 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Typical | †7½ minutes |
| 7 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Typical | †3½ minutes |
| 8 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Severe symptoms, falls on side. Apnoea; on feet | Severe shock followed by recovery |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE IV

*Passive Anaphylaxis with Anti-Galacto-Globulin Serum*

Effect of Uncombined Galactoside

| Guinea Pig No. | Anti-galacto-globulin serum i.p. | Interval between injection of serum and anaphylactic test | Injection of glucoside i.v. | Result | Interval between injection of glucoside and sugar-protein | Injection of sugar-protein i.v. | Symptoms | Result |
|---|---|---|---|---|---|---|---|---|
| | *cc.* | *hrs.* | *galactoside* | | *hrs.* | *galacto-egg-albumin* | | |
| 1 | 5 | 24 | 1 cc. | No reaction | 2 | 1 cc. | Marked scratching, coughing, backing, respiratory distress | Definite symptoms followed by recovery |
| 2 | 5 | 24 | 1 cc. | No reaction | 2½ | 1 cc. | Typical | †4 minutes |
| 3 | 5 | 24 | 1 cc. | No reaction | 2½ | 1 cc. | Typical | †3½ minutes |
| 4 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | None | No reaction |
| 5 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | None | No reaction |
| 6 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | None | No reaction |
| | | | *glucoside* | | | | | |
| 7 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Typical | †2½ minutes |
| 8 | 5 | 24 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Typical | †3 minutes |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE V

*Desensitization Induced in Passively Sensitized Guinea Pigs by Injection of Glucoside and Homologous Sugar-Protein*

| Guinea Pig No. | Serum used for passive sensitization i.p. | Previous tests. Injections made 24 hrs. after serum i.v. | Result | Interval between previous tests and desensitization tests | Material injected i.v. | Symptoms | Result |
|---|---|---|---|---|---|---|---|
| | | | | *hrs.* | | | |
| 1 | 5 cc. anti-gluco-globulin serum | 1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin | No reaction | 4½ a | 1 cc. gluco-egg-albumin | None | No reaction |
| 2 | 5 cc. anti-gluco-globulin serum | 1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin | No reaction | 4½ | 1 cc. gluco-egg-albumin | None | No reaction |
| 3 | 5 cc. anti-gluco-globulin serum | 1 cc. glucoside followed immediately by 1 cc. gluco-egg-albumin | No reaction | 4½ | 1 cc. gluco-egg-albumin | None | No reaction |
| 4 | 5 cc. anti-galacto-globulin serum | 1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin | No reaction | 1 | 1 cc. galacto-egg-albumin | None | No reaction |
| 5 | 5 cc. anti-galacto-globulin serum | 1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin | No reaction | 2 | 1 cc. galacto-egg-albumin | None | No reaction |
| 6 | 5 cc. anti-galacto-globulin serum | 1 cc. galactoside followed immediately by 1 cc. galacto-egg-albumin | No reaction | 3½ | 1 cc. galacto-egg-albumin | None | No reaction |

i.p. = intraperitoneal.
i.v. = intravenous.

The results are, in every respect, identical with those given in Table III both with regard to the transitory nature of the phenomenon and to its specificity.

The mechanism of the protection afforded by the glucosides is not as yet understood. The fact that the protective effect is no longer demonstrable after two hours indicates that "desensitization,"—if such has occurred—is transitory. Instances of what appears to be true desensitization have been observed in these experiments and are recorded in Table V.

Guinea pigs Nos. 1, 2, and 3, passively sensitized with antigluco-globulin serum, were protected from the shocking effect of 1 cc. of gluco-albumin by a previous injection of homologous glucoside. Four and one-half hours later the same pigs received a second injection of 1 cc. of gluco-albumin. No reaction occurred. The absence of shock following the second injection of whole antigen seems to be dependent upon the first dose of gluco-albumin. That the glucoside alone plays no direct part in the refractory state is demonstrated by its ineffectiveness when injected singly, two hours prior to the shocking dose (Table III). Pigs Nos. 4, 5, and 6 of Table V demonstrate the same principle, the difference being that antigalacto-globulin serum was used for sensitization, and galactoside and galacto-albumin were employed to complete the test.

### *C. Results obtained with uncombined protein*

Avery and Goebel (2) have shown that the serum of rabbits immunized with synthetic sugar-proteins (gluco- or galacto-globulin) possesses two distinct antibodies; 1) the specific precipitin so intimately associated with the carbohydrate radical of the compound; 2) the "common" precipitin, reactive with globulin alone. Passive anaphylaxis experiments were, therefore, carried out, using pure horse globulin as the toxigenic material.

For sensitization, one pig received intraperitoneally 1 cc. of serum prepared by immunization with gluco-globulin; a second pig received 1 cc. of serum derived from a rabbit immunized with galacto-globulin. Twenty-four hours later each received 12 mgms. of globulin. Both animals died in typical anaphylactic shock (Table VI). A normal pig receiving the same dose of globulin gave no reaction.

TABLE VI

*Passive Anaphylaxis with Anti-Gluco-Globulin and Anti-Galacto-Globulin Sera*

Reactions induced by the use of horse globulin

| Guinea pig No. | Sensitizing serum i.p. | Time interval | Shocking dose i.v. | Symptoms | Result |
|---|---|---|---|---|---|
| | | *hrs.* | | | |
| 1 | 1 cc. anti-gluco-globulin serum | 24 | 1 cc. horse-globulin (12 mgms.) | Typical | †3 minutes |
| 2 | 1 cc. anti-galacto-globulin serum | 24 | 1 cc. horse-globulin | Typical | †2½ minutes |
| 3 | Normal control | — | 1 cc. horse-globulin | None | No reaction |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

### *Active Anaphylaxis*

For purposes of testing the capacity of the synthetic sugar-proteins to produce active sensitization, 10 guinea pigs were injected intraperitoneally with 5 cc. of gluco-globulin and 10 other animals were similarly inoculated with 5 cc. of galacto-globulin.

In the preparations employed, 5 cc. of sugar-globulin contained 50 mgms. of protein. It is estimated (1) that 15 per cent by weight of this complex represents chemically combined glucoside. Consequently each pig received approximately 7.5 mgms. of the synthesized sugar-protein. The 20 pigs were tested 21 days later for active sensitization. As in the experiments on passive anaphylaxis, the sensitizing dose consisted of material in which the glucoside was joined to a protein heterologous to that used for sensitization. As previously mentioned, this precaution was taken in order to eliminate the possibility of protein-antiprotein reactions entering into the results.

In Table VII, the results of active sensitization obtained by the use of gluco-proteins are given.

Pigs Nos. 1, and 2, previously sensitized with gluco-globulin, were injected intravenously with 1 cc. of gluco-egg-albumin. Each promptly succumbed with typical symptoms. Pigs Nos. 3 and 4 of Table VII demonstrate the specificity of the sensitization; both of the animals when tested with 1 cc. of galacto-albumin showed no reaction. However, when, 3 hours later, 1 cc. of gluco-albumin was introduced, they reacted fatally. Pigs Nos. 5, 6, 7, and 8 were used to determine the influence of homologous uncombined glucoside on the reaction. In these tests the same relations were found to exist as described in the experiments on passive

TABLE VII

*Active Anaphylaxis in Guinea Pigs Sensitized with Gluco-Globulin*

| Guinea Pig No. | Sensitizing dose i.p. | Interval between sensitizing injection and test | 1st Injection | | Interval between 1st and 2nd injection | 2nd Injection | | Interval between 2nd and 3rd injection | 3rd Injection | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Material i.v. | Result | | Material i.v. | Result | | Material i.v. | Result |
| | *gluco-globulin* | *days* | *gluco-egg-albumin* | *min.* | *hrs.* | | *min.* | *hrs.* | | *min.* |
| 1 | 5 cc. | 21 | 1 cc. | †5 | — | — | — | — | — | — |
| 2 | 5 cc. | 21 | 1 cc. | †3½ | — | — | — | — | — | — |
| | | | *galacto-egg-albumin* | | | *gluco-egg-albumin* | | | | |
| 3 | 5 cc. | 21 | 1 cc. | No reaction | 3 | 1 cc. | †3 | — | — | — |
| 4 | 5 cc. | 21 | 1 cc. | No reaction | 3 | 1 cc. | Severe symptoms. Recovery | — | — | — |
| | | | *glucoside* | | | | | | | |
| 5 | 5 cc. | 21 | 1 cc. | No reaction | 1½ | 1 cc. | †3 | — | — | — |
| 6 | 5 cc. | 21 | 1 cc. | No reaction | 2 | 1 cc. | †2½ | — | — | — |
| 7 | 5 cc. | 21 | 1 cc. | No reaction | 2 | 1 cc. | †4½ | — | — | — |
| 8 | 5 cc. | 21 | 1 cc. | No reaction | Followed immediately | 1 cc. | No reaction | — | — | — |
| | | | *galactoside* | | | *galacto-egg-albumin* | | | *gluco-egg-albumin* | |
| 9 | 5 cc. | 21 | 1 cc. | No reaction | 4 | 1 cc. | No reaction | ½ | 1 cc. | †4½ |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

TABLE VIII

*Active Anaphylaxis in Guinea Pigs Sensitized with Galacto-Globulin*

| Guinea Pig No. | Sensitizing dose i.p. | Interval between sensitizing injection and test | 1st Injection | | Interval between 1st and 2nd injection | 2nd Injection | | Interval between 2nd and 3rd injection | 3rd Injection | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Material i.v. | Result | | Material i.v. | Result | | Material i.v. | Result |
| | *galacto-globulin* | *days* | *galacto-egg-albumin* | *min.* | *hrs.* | | *min.* | *hrs.* | | *min.* |
| 1 | 5 cc. | 21 | 1 cc. | †3½ | — | — | — | — | — | — |
| 2 | 5 cc. | 21 | 1 cc. | †4 | — | — | — | — | — | — |
| | | | *gluco-egg-albumin* | | | *galacto-egg-albumin* | | | | |
| 3 | 5 cc. | 21 | 1 cc. | No reaction | 2½ | 1 cc. | †4 | — | — | — |
| 4 | 5 cc. | 21 | 1 cc. | No reaction | 2 | 1 cc. | †3 | | | |
| | | | *galactoside* | | | | | | | |
| 5 | 5 cc. | 21 | 1 cc. | No reaction | 1½ | 1 cc. | †4½ | | | |
| 6 | 5 cc. | 21 | 1 cc. | No reaction | 3 | 1 cc. | †3 | | | |
| 7 | 5 cc. | 21 | 1 cc. | No reaction | 3 | 1 cc. | †2 | | | |
| 8 | 5 cc. | 21 | 1 cc. | No reaction | Followed immediately by | 1 cc. | Very slight symptoms. Recovery | | | |
| | | | *glucoside* | | | *gluco-egg-albumin* | | | *galacto-egg-albumin* | |
| 9 | 5 cc. | 21 | 1 cc. | No reaction | 4 | 1 cc. | No reaction | 1 | 1 cc. | †5 |
| 10 | 5 cc. | 21 | 1 cc. | No reaction | 4 | 1 cc. | No reaction | 1 | 1 cc. | †3½ |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

anaphylaxis. When glucoside was injected immediately before gluco-albumin, complete inhibition of anaphylaxis resulted. However, when glucoside was injected one and one-half to two hours before the shocking dose (Pigs Nos. 5, 6, and 7), no protection occurred. Pig No. 9 of Table VII is further evidence of the specificity of active sensitization; in this animal attempts to inhibit shock with heterologous galactoside and to desensitize with galacto-albumin were ineffectual since the subsequent injection of gluco-albumin produced characteristic death.

TABLE IX

*Active Anaphylaxis in Guinea Pigs Sensitized with Gluco-Globulin and Galacto-Globulin*

Reactions induced by the use of horse globulin—the protein common to both antigens

| Guinea pig No. | Sensitizing serum i.p. | Time interval | Shocking dose i.v. | Symptoms | Result |
|---|---|---|---|---|---|
| | | *days* | | | |
| 1 | 5 cc. gluco-globulin | 21 | 1 cc. horse-globulin (18 mgms.) | Typical | †3½ minutes |
| 2 | 5 cc. gluco-globulin | 21 | 0.5 cc. horse-globulin (9 mgms.) | Typical | †14 minutes |
| 3 | 5 cc. galacto-globulin | 21 | 1 cc. horse-globulin (18 mgms.) | Typical | †3 minutes |
| 4 | 5 cc. galacto-globulin | 21 | 0.5 cc. horse-globulin (9 mgms.) | Typical | †7½ minutes |
| 5 | Normal control | — | 1 cc. horse-globulin (18 mgms.) | None | No reaction |

† Death of animal.
i.p. = intraperitoneal.
i.v. = intravenous.

In Table VIII, a similar group of experiments was carried out employing galacto-globulin for sensitization instead of gluco-globulin. Galacto-albumin was the toxigenic agent; galactoside was injected for inhibition tests. Results comparable in every respect to those recorded in Table VII were obtained. Consequently a detailed description need not be given.

Table IX presents the results obtained in guinea pigs actively sensitized with gluco-globulin (animals Nos. 1 and 2) or with galacto-globulin (Nos. 3 and 4) and subsequently injected with horse globulin.

All the animals gave typical reactions. Active sensitivity in these pigs was in all probability induced by the uncombined globulin present in the sensitizing material.

## DISCUSSION

The experiments reported in this paper demonstrate the capacity of artificially prepared sugar-proteins to produce both active and passive anaphylaxis. The tests were devised and carried out in such a manner as to emphasize the significance of the carbohydrate radical. The fact that guinea pigs, passively sensitized with antigluco-globulin serum, or actively sensitized with gluco-globulin, can be subsequently shocked with gluco-albumin, demonstrates that the antigen-antibody specificity in these instances is directly dependent upon the carbohydrate fraction of the antigenic compounds.

The introduction of the sugar radical into the protein molecule endows the new complex with a sharply defined specific antigenicity. This fact is brought out by experiments in which galactoside was substituted for glucoside in the preparation of sugar-proteins used for sensitization. The same specific relations hold in the production of anaphylaxis with galacto-proteins as that described for gluco-proteins. Attempts to incite anaphylactic shock with heterologous material were ineffectual. The results of the anaphylactic experiments conform to the results anticipated by the serological findings of Avery and Goebel (2).

Landsteiner (7), employing complex antigens, has reported experiments on anaphylaxis of a similar character to those presented in this report. He found that guinea pigs sensitized with one azoprotein could be shocked by the injection of a second compound containing the same azo-groups attached to a different protein.

In addition to the new specificity which the carbohydrate radical confers upon the conjugated proteins, the uncombined glucosides by themselves also exert a definite influence on the reactivity of sensitized animals. When sensitized pigs are injected with the homologous glucoside immediately before the introduction of the toxigenic sugar-protein, they are completely protected from shock. However, the protection afforded by the glucoside alone is apparently only transitory; for, when the interval between introduction of glucoside and

shocking agent was as long as two hours, the injection of homologous sugar-protein produced prompt and typical anaphylactic death. That the temporary protection just mentioned is specific, was demonstrated by the experiments in which uncombined carbohydrate of heterologous type was shown to exert no such protective action.

The transitory, specific protection afforded by the glucosides alone is not yet understood. Landsteiner (7) found in experiments on anaphylaxis with azoproteins that the azo-component, when injected one hour before the conjugated azoprotein, inhibited shock. He considered that a state of anti-anaphylaxis had been induced. In the tests with glucosides and sugar-proteins, sufficient evidence has not been obtained to interpret the mechanism other than to say that the inhibitory effect of the glucosides disappears in at least two hours.

Active and passive anaphylaxis has also been elicited with uncombined globulin. Whether animals were passively sensitized with anti-gluco-globulin or antigalacto-globulin serum, the toxigenic action of globulin was equally effective. Since the sera employed contained anti-globulin antibodies, these results were to be expected. Guinea pigs actively sensitized with either gluco-globulin or galacto-globulin, were found to be equally sensitive to uncombined globulin. Since the material used to produce active sensitization contained free globulin, the subsequent intoxication with horse globulin is obviously based on a simple protein- anti-protein reaction.

## CONCLUSIONS

1. Guinea pigs passively sensitized with the serum of rabbits immunized with an artificially prepared sugar-protein (gluco-globulin) exhibit typical anaphylactic shock when subsequently inoculated with gluco-albumin; the serum of rabbits immunized with a second synthetic sugar-protein (galacto-globulin) similarly sensitizes guinea pigs to galacto-albumin. The reactions, in each instance, are specific and depend for their specificity on the carbohydrate component, and not on the protein fraction of the synthesized sugar-protein.

2. Guinea pigs actively sensitized with gluco-globulin or galacto-globulin are similarly subject to anaphylactic shock, when injected, after 21 days, with sugar-proteins containing carbohydrate identical with that present in the sensitizing antigen, regardless of the kind of protein with which it is combined.

3. The unconjugated glucosides, although themselves not capable of inducing shock, inhibit the anaphylactic reaction when injected immediately prior to the introduction of the toxigenic sugar-protein. The protective action of the glucosides disappears within two hours after injection. In order to elicit the phenomenon, the carbohydrate must be the same as that combined in the sugar-protein complex.

4. Anaphylactic shock may be induced by uncombined globulin in guinea pigs passively sensitized with either antigluco-globulin serum or antigalacto-globulin serum; globulin is similarly effective in animals actively sensitized with gluco-globulin or galacto-globulin. The reactions elicited by globulin alone are dependent upon the common protein present in the antigens, and exhibit only species specificity.

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# THE LIVER AS THE SOURCE OF FIBRINOGEN

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The present work embodies studies on the problem of fibrin regeneration in rabbits deprived of the entire liver (1).

In the past the formation of fibrinogen has been ascribed to various organs. Claude Bernard in 1848 (2), Lehmann (3), and Brown-Séquard (4) believed they had found the blood of the mesenteric veins rich in the substance and the content of the hepatic and renal veins relatively poor. Years later these findings seemed to be confirmed by the work of Dastre (5–8). As result the liver was credited with the function of fibrinogen destruction while certain other organs, particularly the intestine, were looked upon as its chief site of origin. By others, in the meantime, the lungs and skin (6), the bone marrow (9, 10), and leucocytes (11) have been thought to form it. The more important of these views will be considered further on in connection with our findings.

In 1905 Doyon (12, 13) and his associates found that extensive degenerative changes in the liver were accompanied by a fall in the blood fibrinogen content of dogs poisoned by chloroform and phosphorus. They suggested the liver as a probable source of fibrinogen. In the last quarter of a century this view has steadily gained acceptance. Nearly all workers on the question of the origin of fibrinogen are now agreed that the liver is its chief source. Whipple and Hurwitz (14) have determined a striking correspondence between the extent of liver damage and the decrease of blood fibrinogen in dogs poisoned by chloroform and phosphorus, and they have suggested an hepatic origin of this blood protein. Goodpasture (15), studying fibrinogen regeneration in dog's blood, agreed with this view in part but concluded that the intestines act as a controlling if unessential factor in fibrinogen formation. More will be said of this view further on.

It remained for Meek (16) to demonstrate clearly a rôle for the liver in fibrinogen formation. This author proved that the substance is regenerated in the Eck fistula dog. But if ligation of the hepatic artery was practiced in addition and the occlusion of the portal vein made close to the liver after formation of the Eck fistula, no regeneration whatever occurred. Indeed there followed a depletion of the amount already present in the blood.

In a series of studies on fibrin metabolism in the dog, under normal and pathological conditions, Foster and Whipple (17–20) have recently brought further

evidence to show that the liver is the chief if not the sole source of fibrinogen formation, a view strengthened by the work of Schultz, Nicholes and Schaefer (21).

From the foregoing it is clear that the studies of recent years have been converging upon the liver as the source of fibrinogen. But certain cogent objections have prevented a definite conclusion in the matter. Much of the evidence for a liver origin of fibrinogen has been obtained by injuring the organ with chloroform, phosphorus or carbon tetra chloride. To accept this evidence one must assume that the drugs act only upon the liver, not elsewhere in the body. Williamson and Mann's (22) observations upon hepatectomized animals have clearly shown that this is not the case.

It has seemed wise to us to study again the problem of fibrin regeneration in liverless animals not subjected to the effects of poisons and not suffering from circulatory obstruction to organs other than the liver (1).

### *Fibrinogen Regeneration in the Normal Rabbit*

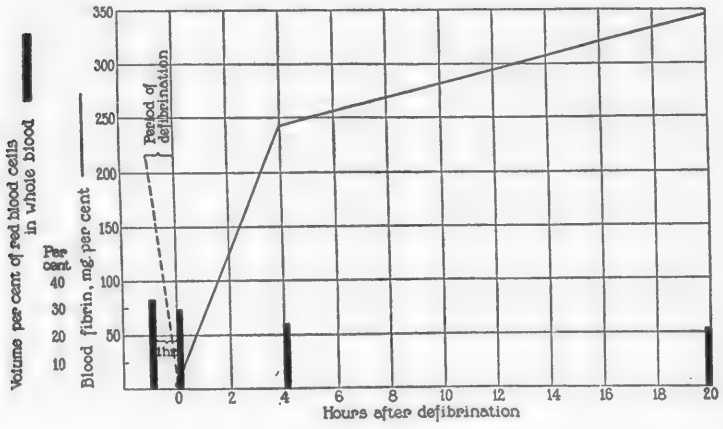
The span of life of the hepatectomized rabbit is short, on the average 24 to 30 hours (1, 23). Before proceeding to studies on the liverless animal it became necessary to estimate the rate and extent of fibrinogen regeneration during such a period in the normal animal.

*Method.*—Subtotal defibrination was carried out in the classical manner in four normal rabbits of about 2 kilograms each. Under ether anesthesia, cannulae were placed in the left carotid artery and jugular vein, and a sample of blood withdrawn for fibrin determination. A supply of blood roughly equivalent to the animal's estimated blood volume, already obtained from donor rabbits, was defibrinated after cross agglutination tests had ensured its compatibility with that of the experimental animal. The defibrinated blood was slowly injected through the cannula inserted in the jugular vein while at the same time an equal volume of arterial blood was removed from the carotid. This, defibrinated in turn, was reinjected into the animal during the removal of another equivalent volume of blood. Five or six repetitions of the procedure reduced the circulating fibrinogen to less than 5 per cent of its original amount. Blood specimens taken at intervals thereafter and examined for fibrinogen content disclosed the rate of its regeneration.

Using duplicate specimens, fibrinogen was estimated in the form of fibrin by the method of Foster and Whipple (17), as modified by Schultz, Nicholes and Schaefer (21). To ensure the complete precipitation of fibrin by the presence of ample thrombin, controls were run, as routine, to which fresh serum had been added. These controls showed no increased amounts of fibrin.

In normal rabbits fibrinogen regeneration was exceedingly rapid. Within five or six hours after a 90 per cent reduction of the substance

in the circulating blood a complete return to the previous amount was observed. Text-figs. 1 and 2 depict the findings in two of the four experiments. In the first one the blood fibrinogen had returned to the normal amount in less than 4 hours after total defibrination and 20 hours later had exceeded this by approximately 50 per cent. In the second instance a slightly less pronounced rise in blood fibrinogen during the first 5 hours was followed by a progressive increase to more than the normal amount in 21 hours and to double the original amount




TEXT-FIG. 1

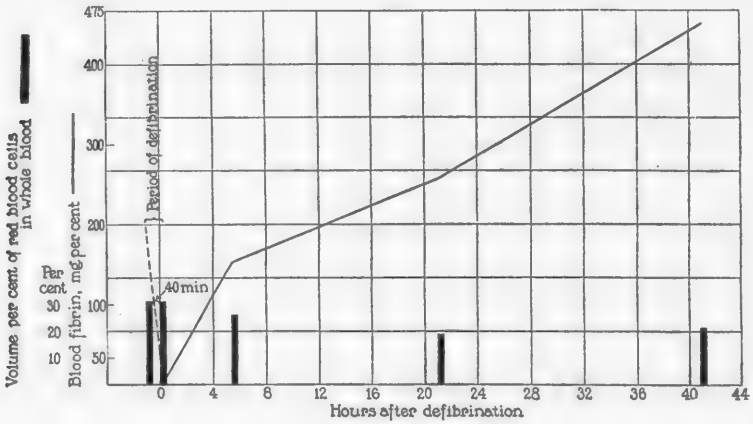
by the second day. The findings in the remaining two experiments were comparable to these in every way.

*Influence of the Absence of the Liver on Fibrinogen Regeneration*

The findings were wholly different in the liverless rabbit. Instead of a rapid fibrinogen regeneration, after partial defibrination, a speedy decrease in the small amount still remaining was the invariable rule.

Under ether anesthesia 10 rabbits of about 2 kilos body weight were subjected to a modified Markowitz operation (24) for inducing a collateral circulation about the liver, as already described by one of us (1). For two months thereafter they were kept on a full mixed diet on which they thrived, as evidenced by a gain in body weight. Then, after a fast of 48–72 hours, hepatectomy (1) was performed under ether and cannulae were placed in the left carotid artery and jugular vein.

In all the experiments blood specimens were drawn from the cannula placed in the carotid artery 10–15 minutes after hepatectomy. Partial defibrination of the circulating blood was next accomplished by the method described in the experiments on normal animals, save that it was not carried quite do far. The procedure accomplished a reduction of approximately 50–70 per cent of the circulating fibrinogen. Ten to fifteen minutes after completion of the partial defibrination another blood specimen was invariably taken. Further specimens were obtained at various time intervals in the different instances, as the charts



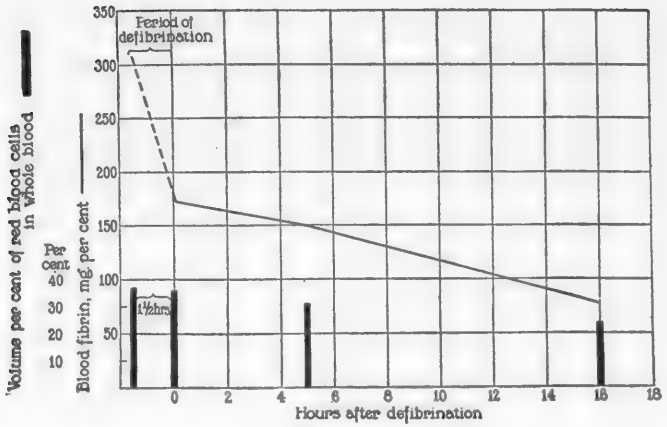

TEXT-FIG. 2

*The Rapid Rate of Fibrinogen Regeneration in the Normal Rabbit*

Text-figures 1 and 2 depict the rate of fibrinogen regeneration in the normal rabbit after almost complete defibrination of the circulating blood, as described in the text. It was exceedingly rapid. The line represents the concentration of fibrinogen in the blood after defibrination; the dark columns indicate the volume per cent of red blood cells in whole blood.

show, and the fibrin determinations carried out as in the preceding experiments. In the specimens taken after defibrination, controls were again run to which serum was added to make up for a possible thrombin lack. This addition did not appreciably increase the amount of fibrin clot obtained, showing that the fall in blood fibrinogen encountered was due to a lack of this protein and not to a fibrin ferment (thrombin) deficiency.

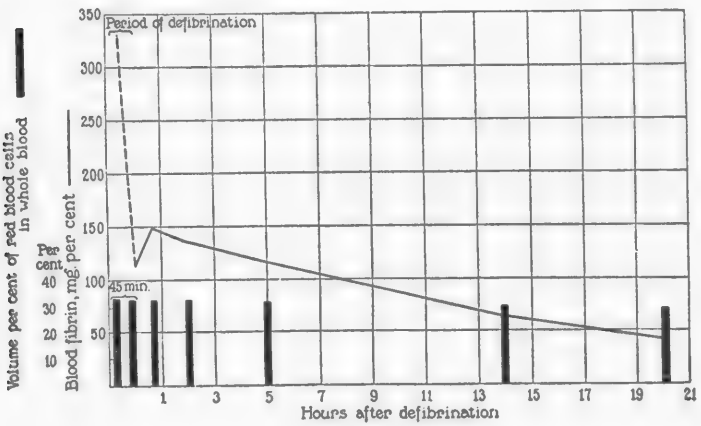
No evidences of fibrinogen regeneration appeared after hepatectomy in any of the experiments. Three typical examples, charted in


Period of defibrination
Volume per cent of red blood cells in whole blood
Per cent
Blood fibrin, mg per cent
1½hrs.
Hours after defibrination

TEXT-FIG. 3

*Failure of Fibrinogen Regeneration in the Hepatectomized Rabbit*

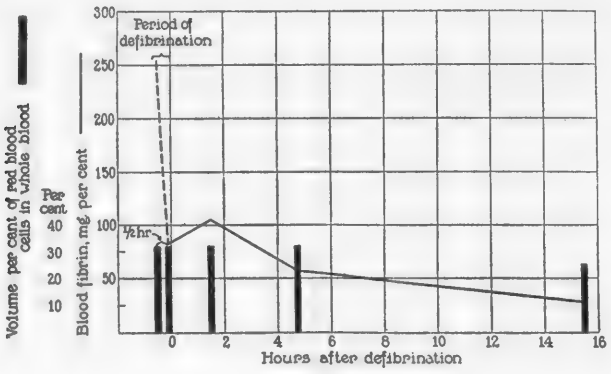
In Text-figures 3, 4 and 5 are charted the changes in blood fibrinogen of hepatectomized rabbits after partial defibrination of the circulating blood. The curves, which are fully discussed in the text, demonstrate the failure of fibrinogen regeneration in the liverless rabbit.


Period of defibrination
Volume per cent of red blood cells in whole blood
Per cent
Blood fibrin, mg per cent
45 min.
Hours after defibrination

TEXT-FIG. 4

Text-figs. 3 to 5, show this fact well. Not only was there no rapid new formation of fibrinogen, such as occurs in the normal rabbit, but a swift fall in the amount of the substance in the blood signified a rapid utilization of the fibrinogen remaining in the organism.

The curves plotted in the text-figures differ in some details but the general findings are the same, and the variations can be safely attributed to the different time intervals at which blood specimens were taken.




TEXT-FIG. 5

*Blood Fibrinogen after Partial Defibrination in the Liverless Rabbit*

In the experiment plotted in Text-fig. 3, the fibrinogen content of the blood apparently decreased progressively, for a third specimen taken 5 hours after defibrination showed slightly less fibrin than the preceding one, and a fourth removed 16 hours later contained but half as much. In later experiments blood specimens taken at shorter intervals after defibrination showed a transient rise in the quantity in the blood, a phenomenon indicative of a fibrin reserve within the body as already noted by Foster and Whipple (20). The rapidity with which this occurred (Text-fig. 4) suggested an inflow of the substance with the lymph. In this experiment the third blood sample was obtained 35 minutes after the second, and a fourth an hour later. The fourth contained less fibrinogen than the third and each subsequent

blood specimen contained progressively less. Three similar experiments not plotted in the text-figures yielded like findings, no increase in fibrinogen being observed in the blood of hepatectomized rabbits later than 45 minutes after defibrination, that is to say after a transient rise. It is to be noted in Text-fig. 4 that the concentration of blood fibrinogen 5 hours after defibrination was still slightly above the figure obtained immediately following this procedure, that is to say in the second blood specimen. In Text-fig. 5 the plotted findings of

TABLE I

*Blood Fibrinogen Content and Percentage of Red Blood Cells in Rabbit's Blood before and after Hepatectomy*

| No. | Preoperative | | Postoperative | | Hours after operation |
|---|---|---|---|---|---|
| | *Fibrinogen, mgs. per cent* | *Volume per cent of red blood cells in whole blood* | *Fibrinogen, mgs. per cent* | *Volume per cent of red blood cells in whole blood* | |
| 1 | 380 | 33 | 130 | 31 | 30 |
| 2 | 408 | 32 | 156 | 34 | 27 |
| 3 | 447 | 36 | 222 | 33 | 27 |
| 4 | 345 | 34 | 201 | 33 | 24 |
| 5 | 262 | 30 | 115 | 32 | 16 |
| 6 | 426 | 30 | 282 | 28 | 15 |
| 7 | 310 | 35 | 171 | 34 | 15 |
| Control Experiment. Ablation of 70 Per Cent of the Liver | | | | | |
| 1 | 246 | 34 | 239 | 35 | 24 |

another experiment show the amount of fibrinogen in the fourth blood specimen, taken 5 hours after defibrination, to be distinctly less than in the second specimen which was removed a few minutes after defibrination. Such an experiment, without the determination shortly after hepatectomy, would have yielded a curve like that given in Text-fig. 3.

The investigation of the source of the fibrinogen reserve has not been attempted. Unfortunately fibrin determinations require so much plasma that frequent sampling is precluded and one is unable to follow at short intervals the changes in concentration of this substance in the blood of any one animal.

### *Blood Fibrinogen after Hepatectomy without Defibrination*

In seven hepatectomized rabbits, which were employed for other experiments which involved no other complicating factors, the blood fibrinogen was estimated prior to removal of the liver and again 15–30 hours later. The results summarized in Table I are consistent. In the absence of the liver the fibrinogen concentration of the circulating blood rapidly decreased in all these animals while the hematocrit readings showed no significant changes. The findings are included here as further evidence not only of the lack of fibrinogen regeneration in the hepatectomized organism but of its destruction.

In a single experiment, also summarized in Table I, blood fibrinogen estimations were made before removal of 70 per cent of the liver of a rabbit and again 24 hours afterwards. As is well known, this procedure fails to induce clinical signs of liver insufficiency in the rabbit (25, 26). The blood fibrinogen showed no decrease.

## DISCUSSION

It is certain from our results that the liver is absolutely essential to the maintenance of the normal quantity of fibrinogen in the blood. But is the liver the only source of fibrinogen? This question has not been answered by these experiments, for they do not preclude the possibility of an inconsiderable extra hepatic formation of fibrinogen, one wholly insufficient to make up for its destruction in the course of normal events. An enormous over production of fibrinogen took place in the normal animals stimulated to regeneration of the substance by defibrination of the blood. Surely a similar activity should have been observed in liverless rabbits after defibrination had there been any other important source of fibrinogen besides the liver. And any considerable compensation should have prevented the speedy decrease of the substance in the blood.

### *Theories of Extra-Hepatic Fibrin Formation*

In the past many theories of extra hepatic fibrin formation have been offered. A little will be said of these.

The work of Mathews (11) and others (15) has tended to show an important activity of the intestinal tract in the origin of fibrinogen. At the present time it is evident that the findings of these authors were due to functional derangement

of the liver and not to injury or removal of the intestine. The experiments (11) had involved surgical interference with the blood supply of the intestinal tract, by ligations of the coeliac axis and of the superior and inferior mesenteric arteries. In addition ablation of segments of the intestine, or total extirpation of the organ, was practiced. For example, in one experiment (11) removal of the intestine from pylorus to rectum led to a pronounced fall in blood fibrinogen. In another experiment involving partial removal of the intestine in a cat, the stomach, pancreas, spleen and a segment of small intestine were allowed to remain. The animal, surviving 5 hours, regenerated fibrin but only half as rapidly as the normal cat. Procedures such as these reduced the circulation through the liver considerably.

The finding of fibrinogen in the blood of the mesenteric veins in greater concentration than in the general circulation has repeatedly been stressed in the literature as evidence for an intestinal origin of the protein (2, 3, 4, 11). The figures presented by the advocates of this view show changes sufficiently small to be accounted for by alterations in the concentration of the blood. The findings lie well within the errors of the methods used.

Müller (9) working with infections in guinea pigs found an increase in the fibrinogen content of the marrow in these animals and concluded that the marrow furnished this protein. In the light of more recent pathological knowledge these conclusions seem unwarranted. It is now well known from the work of Foster and Whipple and others (20, 11) that almost any infection or inflammation in the body leads to fibrinogen increase in the blood and accumulations of fibrin in various locations. Morawitz and Rehn (10) after withdrawing blood, defibrinating and reinjecting it into animals found myelocytic proliferation in the marrow and spleen and believed that this activity showed that fibrin deficiency was being made up in these regions. It is now recognized that red blood cells become fragmented and partially hemolyzed by the process of removal and defibrination. The myelocytic proliferation may have been a secondary consequence of this destruction.

One further point deserves mention. The experiments here described serve well to demonstrate the rapid rate of fibrinogen utilization, a phenomenon already emphasized by Foster and Whipple (19). It indicates an important function of the protein as yet unknown but worthy of investigation. As our experiments have indicated, absence of the liver brings about a true fibrinogen lack but no deficiency in thrombin (fibrin ferment), for an excess of the latter when added to the blood gave rise to no further coagulum of fibrin.

## SUMMARY

In hepatectomized rabbits a progressive decrease in blood fibrinogen occurs. Partial defibrination in the liverless rabbit invariably

results in a progressive decrease in blood fibrinogen preceded by a temporary and slight rise. No evidence has been secured of fibrinogen regeneration in the absence of the liver. From this it follows that the liver is the essential source of fibrinogen and in all probability the sole one.

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# STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI

## IV. Active Immunization of Mice against Type II Pneumococci by Vaccination with Yeast

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Previous evidence (1) of immunological relationship between a variety of *Saccharomyces cerevisiae* and the Type II Pneumococcus consisted in the inter-reactions of the yeast and the bacteria in the antisera produced by immunization of rabbits with the two different microorganisms. This paper reports the active immunization of mice against Type II pneumococci by vaccination with yeast.

A group of mice were vaccinated with heated suspensions of yeast cells, and tested for active immunity by subsequent injection of virulent Type II Pneumococcus. Because of the known (2) irregularity in the active immunity responses of individual mice to homologous vaccination with Type II pneumococci, it seemed best to base the immunity tests of the yeast-vaccinated mice upon the percentage of animals surviving the test injection of a uniform dose of the Type II bacteria. In order to show that the protection afforded by vaccination with the yeast was not non-specific, groups of yeast-vaccinated mice were tested against comparable doses of Types I and III, as well as against Type II pneumococci.

### EXPERIMENTAL

*Methods:* A group of 100 white mice of approximately the same age and weight were injected subcutaneously at 2 day intervals with 0.5 cc. of a 0.1 per cent. suspension of the dried yeast cells (boiled 5 minutes); after 12 subcutaneous injections and a rest of 3 days, two intraperitoneal injections were given. The active immunity tests were made on the 92 surviving mice 12 days after the last immunization injection.

The yeast-vaccinated animals were divided into three groups: 57 to be tested against Type II pneumococci, 20 against Type I, and 15 against Type III pneumococci. A uniform dosage of 100 times the minimum required for death of normal mice was employed for the tests with the three types of pneumococci. The quantitative sufficiency of the test doses was controlled by injecting groups of normal mice with each culture. The test strains were highly virulent for mice; the cultures used were inoculated directly from the heart's blood of mice. The results are presented in Table I.

The results (Table I) showed that 20 of 57, or 34 per cent of the mice vaccinated with yeast were protected against subsequent infection with 100 lethal doses of virulent Type II pneumococci. The uniform deaths in the control group of non-vaccinated mice proved that the infection test dose was sufficient to eliminate any irregularities

TABLE I

*Specificity of the Protection of Mice against Type II Pneumococcus by Vaccination with Yeast*

| Tests against different types of pneumococcus | Vaccinated mice | | | Normal mice | | |
|---|---|---|---|---|---|---|
| | Number of animals tested | Number survived | Percentage of animals protected | Number of animals tested | Number survived | Percentage of animals survived |
| | | | *per cent* | | | *per cent* |
| Type II | 57 | 20 | 34 | 16 | 0 | 0 |
| Type I | 20 | 0 | 0 | 10 | 0 | 0 |
| Type III | 15 | 0 | 0 | 7 | 0 | 0 |

due to the possible occurrence of individual mice possessing unusual natural immunity against the Type II bacteria. Hence, the vaccinated mice that survived can be considered to have acquired their immunity against Type II pneumococci by virtue of their vaccination with yeast. Active immunity against Type II pneumococci is not easily obtained by mice even when vaccinated with the pneumococci themselves; and it is interesting to note that the percentage of individual mice that obtained anti-Type II immunity in response to the yeast vaccination is about the same as the percentage of mice that obtain immunity in response to homologous vaccination with the Type II bacteria (2). The uniform death of the yeast-vaccinated mice tested against Types I and III pneumococci is important evi-

dence that the protection conferred by the yeast vaccination was specific to the Type II Pneumococcus.

## COMMENT

The described protection of mice indicates that these animals respond to yeast antigen with production of antibodies specifically reactive with Type II pneumococci. It had been shown (1) that rabbits can respond to the same yeast antigen with production of antibodies that agglutinate Type II bacterial cells, precipitate Type II culture filtrates, and confer passive protection against Type II infection; the more responsive rabbits yielding sera comparable in anti-Type II potency to the antisera produced by immunization with the Type II bacterial cells themselves. In another study (3) it was found that the relative antigenic effectiveness of two related strains of pneumococci differed in different species of animals. The present report therefore, is of interest as evidence that the immunological relationship between the yeast and Type II pneumococci causes mice, as well as rabbits, to respond to yeast antigen with production of antibodies specifically reactive with the Type II variety of Pneumococcus.

## SUMMARY

Mice vaccinated with yeast were protected against subsequent infection with Type II pneumococci, but not against Types I or III. While the protection was not universal, as high a percentage of mice acquired active anti-Type II immunity, as was obtained by vaccination of another group of mice with Type II pneumococci themselves. This specific protection of mice by active immunization with the yeast antigen, is probably due to the same immunological relationship responsible for the anti-Type II reactivity of the antisera of rabbits immunized with yeast antigen.

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# INFECTIVITY OF BLOOD DURING THE COURSE OF EXPERIMENTAL YELLOW FEVER

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A problem of importance in the study and control of yellow fever is the period during which a patient may be a source of infection through the bites of mosquitoes.

Work on this question was initiated by members of the American Commission in Cuba (Reed, Carroll, Agramonte and Lazear (1)) who carried the disease from person to person by *Aedes aegypti* which originally became engorged on the first to third days of a patient's illness. Similarly, the French Commission in Brazil (Marchoux, Salimbeni and Simond (2) and Marchoux and Simond (3)) found that yellow fever could be transmitted to volunteers by mosquitoes fed on the first, second and third days of disease and by blood or serum from a patient in the same period. More recently, several workers have been able to study the question of blood infectivity in human cases by the use of *Macacus rhesus* as the experimental host. Bauer and Hudson (4) report that a lot of *A. aegypti* fed on a human case 12 hours after onset of illness transmitted yellow fever to monkeys, while a lot fed 36 hours after onset and the same period before death failed to transmit it, although blood coincidentally withdrawn and injected into another monkey induced a fatal attack. Mathis, Sellards and Laigret (5) infected monkeys by means of the bites of mosquitoes which had been fed upon a patient at the end of the first day of acute illness, and also by the injection of blood withdrawn at the same time. Aragão (6) records the results of injecting monkeys with patients' blood during the recent epidemic in Rio de Janeiro. He was able to infect animals fatally with blood procured at various times up to 72 hours after the beginning of illness, but not later; nor could he transmit the disease by the inoculation of blood and organ emulsions obtained at necropsy.

The foregoing observations indicate a general agreement on the infectivity of blood in human cases during the first three days of illness. Whether the virus is in the circulating blood before the onset of fever has been, of necessity, less readily determined. Marchoux and Simond (3) report that in human beings their attempts to transmit

the virus by means of mosquitoes fed during the incubation period of yellow fever were unsuccessful. Kuczynski (7) has recently stated that the virus does not circulate in the blood during the incubation period, either in man or monkey.

The cardinal features of the human disease are reproduced in *Macacus rhesus*. With the purpose of throwing further light on the nature of the disease and the behavior of the virus, we have attempted to repeat and enlarge upon the experiments just referred to. Other

TABLE I

*The Temperature Record of Monkeys after Infecting*

| Days after inoculation | Monkey A | | Monkey B | | Monkey C | | Monkey D | | Monkey E | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A.M. | P.M. | A.M. | P.M. | A.M. | P.M. | A.M. | P.M. | A.M. | P.M. |
| 1 | 103.5 | 103.5 | 102.8 | 101.8 | 102.8 | 102.8 | 102.1 | 101.9 | 103.1 | 103.1 |
| 2 | 105.5 | 105.5 | 102.2 | 102.2 | 102.0 | 102.6 | 102.3 | 103.1 | 102.2 | 102.5 |
| 3 | 104.9 | 105.9 | 102.0 | 105.1 | 102.0 | 103.6 | 104.3 | 103.8 | 104.1 | 104.2 |
| | | | | | | | | Dead | | |
| 4 | 101.6 | 103.9 | 102.9 | 105.4 | 103.5 | 103.6 | | | 104.2 | 104.3 |
| 5 | 99.2 | 103.8 | 98.9 | 101.8 | 101.9 | 103.8 | | | 99.8 | 102.9 |
| | | | | | | | | | | Dead |
| 6 | 103.1 | 104.9 | 102.0 | 102.2 | 102.5 | 103.3 | | | | |
| | | Dead | | | | | | | | |
| 7 | | | 103.7 | 104.8 | 102.4 | 103.7 | | | | |
| 8 | | | 102.0 | 104.5 | 104.9 | 105.1 | | | | |
| | | | | Dead | | | | | | |
| 9 | | | | | 105.4 | 105.1 | | | | |
| 10 | | | | | 96.9 | 95.0 | | | | |
| | | | | | | Dead | | | | |

advantages to be derived from these experiments are in connection with such laboratory procedures as the transmission of disease, maintenance of strains and, possibly, the preparation of "vaccines."

Five monkeys were infected with the Asibi strain of yellow fever, three by the bites of *A. aegypti* and two by the injection of monkey blood-virus. Attempts were made to transmit the virus from these animals at 24-hour intervals from the time of inoculation until death, by exposing them to batches of *A. aegypti* and by the injection of blood directly into other monkeys. In two instances, the same procedures were followed at the end of the first 12 hours as well. After appro-

priate periods, the mosquitoes were allowed to bite normal monkeys to test their infectivity.

The results are summarized in charts which plot also the highest daily temperatures of each animal; these readings, with only four exceptions, are the afternoon records. The morning and afternoon temperatures are tabulated in Table 1.

## EXPERIMENTATION

*M. rhesus* A was injected with blood-virus, and on the second and third days after inoculation showed a marked febrile reaction. On the following two days, the temperature dropped. A terminal rise occurred on the day of death which was the sixth after inoculation. Each day and immediately postmortem, mosquitoes were fed on this animal and 0.5 cc. and 0.05 cc. blood were injected directly into other monkeys. The necropsy findings in A were typical of experimental yellow fever.

The temperature curve and results of attempts at transmission of disease are presented in Chart 1. This animal proved infective for mosquitoes fed from 24 to 96 hours after inoculation, but subsequent feedings gave negative results. Eleven of the twelve insects originally exposed at the first 24-hour interval failed to produce disease in a test animal 13 days after their original feed, but the same animal died when bitten 11 days later by 9 of these insects. A threatening epidemic in the next lot, fed at the febrile onset, necessitated exposure of the test animal on both the eleventh and fourteenth days after the original engorgement. It is interesting to note that one insect of this lot later caused fatal yellow fever by merely plunging its proboscis through the skin of a test *rhesus*, apparently without obtaining blood. Epidemics among the mosquitoes during the study of this and the following monkey necessitated varied and somewhat short periods of incubation in the insects to obtain a maximum longevity and number of bites. When the monkey was exposed to mosquitoes just postmortem, its peripheral circulation was so impaired that only one insect became engorged by feeding on a dependent part of the body.

Fatal attacks were caused by both doses of blood (0.5 cc. and 0.05 cc.) drawn on the first to fourth days, inclusive, after inoculation. Only the smaller amount on the fifth day induced fatal disease. On the day of death and at necropsy, the subinoculations were negative except for febrile reactions in three of the four monkeys.

*M. rhesus* B was infected by the bites of 9 *A. aegypti* and ran a typical course with death occurring on the eighth day. After 2 days of incubation, the animal registered 2 days of fever, followed by 2 days of low temperature and finally by the same period of thermal elevation. Each day and at the point of death, the monkey was exposed to separate batches of mosquitoes and 0.5 cc. and 0.05 cc.

blood were subinoculated directly into other animals. The animal died of yellow fever, displaying the usual pathologic lesions.

Chart 2 outlines the results of attempts to transmit the disease, in relation to the temperature and course. Mosquitoes fed on the second to fifth days transmitted yellow fever by bite to test animals. The lots fed the days before and after the febrile period failed to induce infection after 13 and 10 days incubation,

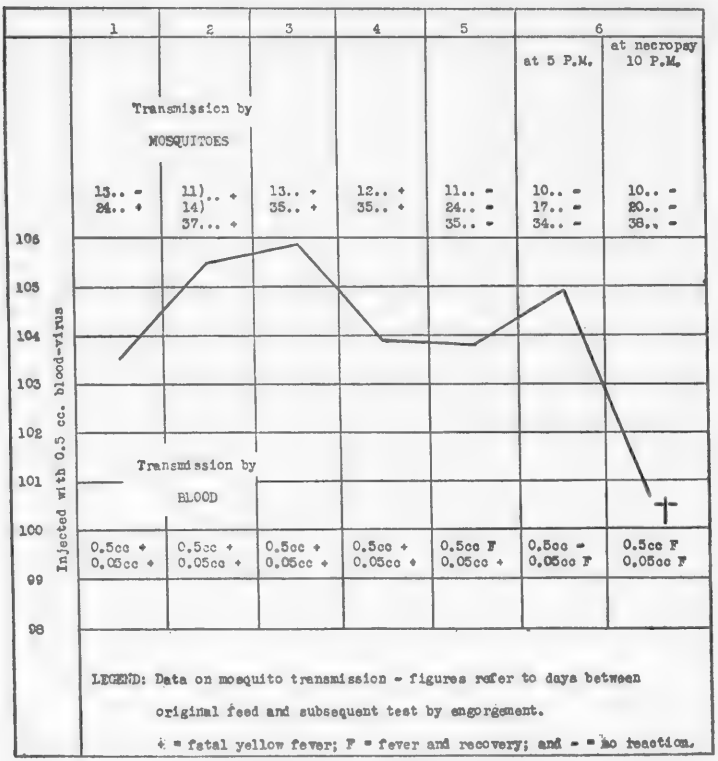


CHART 1. *M. rhesus* A

respectively, but killed the same test animals when fed at 25 and 20 days. The two lots fed during fever transmitted the disease by bite at their first tests, 12 and 10 days after the original infective feed.

At the 24-hour interval after infecting, fatal results were obtained by the subinoculation of 0.5 cc. and not of 0.05 cc. From the second to the seventh day, inclusive, both doses successfully transmitted the disease. At necropsy, the smaller, and not the larger dose, was fatal.

*M. rhesus* C was bitten by 2 *A. aegypti* of an infective lot. The course of disease was quite different from the two preceding monkeys, and yet a course often seen in experimental yellow fever. After 2 days of incubation, the temperature rose to 103.6° to 103.8° on the third to fifth days, followed by a slight drop (103.3° and 103.7°) on the next 2 days. On the eighth and ninth days, the monkey registered temperatures above 105° and on the tenth went into collapse and died. *A. aegypti* were fed and subinoculations were made daily, as

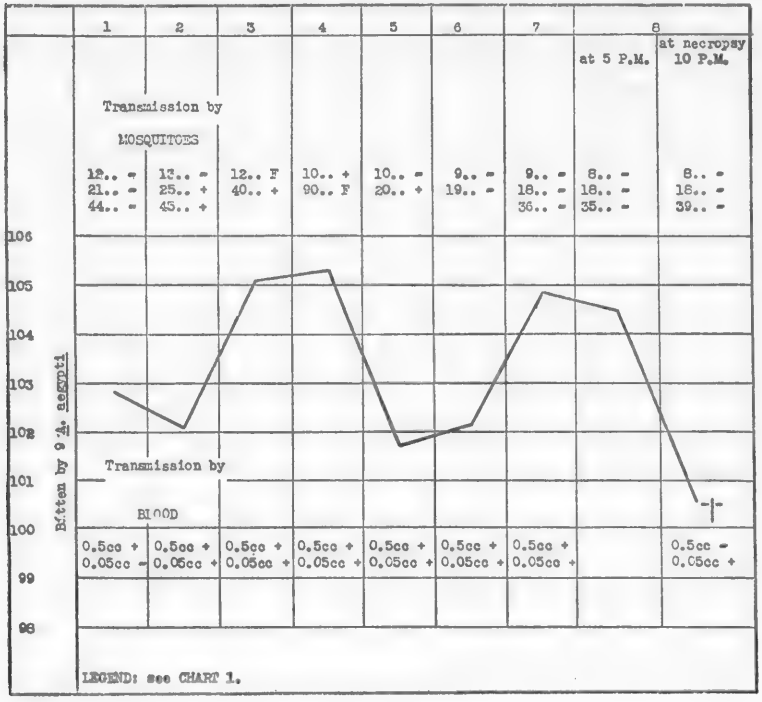


CHART 2. *M. rhesus* B

in the case of the previous monkeys, death occurring just after the last exposure to mosquitoes. The necropsy findings were typical of yellow fever in the monkey.

Chart 3 gives the daily temperature after infecting, as well as for the six preceding days, and the results of attempts at transmission. It is seen that the mosquito lots fed on the second to sixth days acquired infectivity. All of these lots were given a testing feed at 10 and 13 days after their original engorgement and if negative, subsequently at varying intervals. The third-day lot, only,

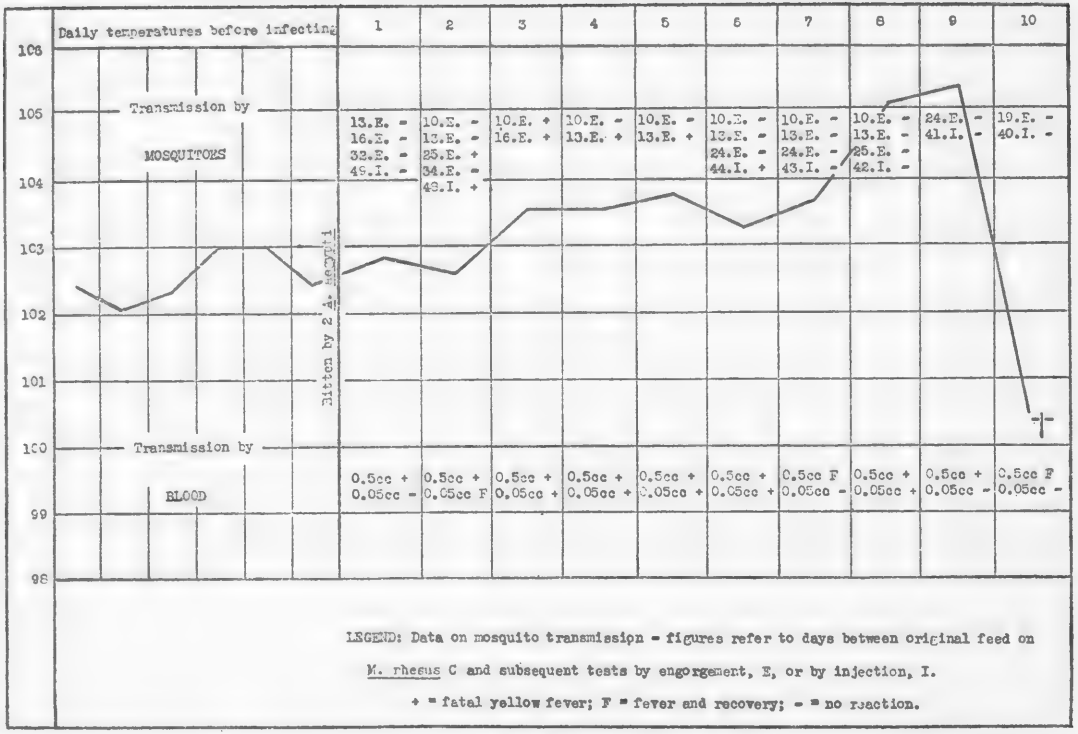


CHART 3. *M. rhesus* C

transmitted the disease at 10 days and the fourth and fifth at 13 days. Five, 10 and 6 insects of the 22 originally engorging on the second day, failed to infect test animals after 10, 13 and 34 days, but 10 of these insects induced fatal yellow fever 25 days after their infecting feed. The sixth-day lot failed to transmit by bite at 10, 13 and 24 days but later produced fatal disease when killed and injected, the number of specimens involved being 15, 9, 10 and 10, respectively.

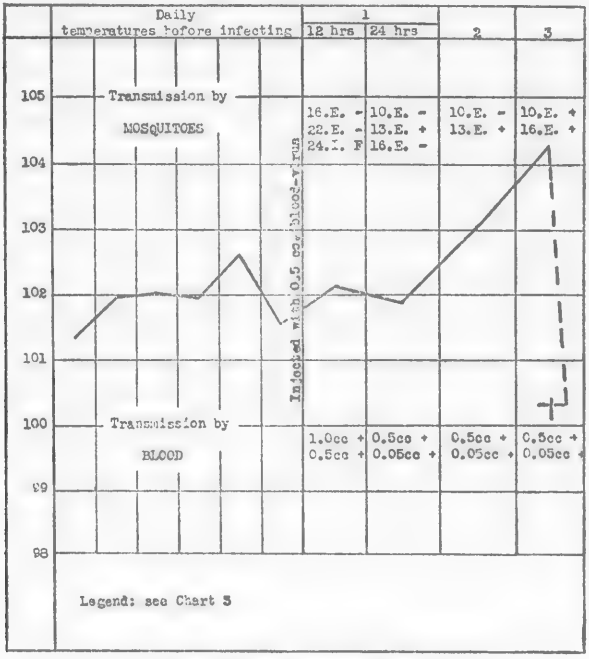


CHART 4. *M. rhesus* D

Only the larger subinoculated dose of blood (0.5 cc.) was fatal on the first 2 days after infecting. From the third to the sixth day both doses transmitted the disease fatally and on the seventh day, both amounts failed to kill. The two subinoculated animals died from the injections of the eighth day, and the monkey injected with the larger dose on the ninth day succumbed. Blood drawn at death was not fatal in either test animal.

*M. rhesus* D was infected by inoculation with monkey blood-virus. The course of the disease was fulminating, the animal dying with typical pathologic findings 82 hours after inoculation. Fever was registered only late in the course.

Transmissions were attempted with mosquitoes and blood at the end of 12 hours, as well as at the usual 24-hour periods.

Chart 4 presents the results in relation to the temperature record of the monkey studied. It will be noted that all lots of insects picked up the virus at each 24-hour feeding, the last being 10 hours before death. Transmission with the first lot was accomplished only by the injection of macerated insects; the injected monkey recovered, but a subinoculated animal died of yellow fever. By feeding 33 insects of the 24-hour batch after 13 days incubation, the disease was fatally

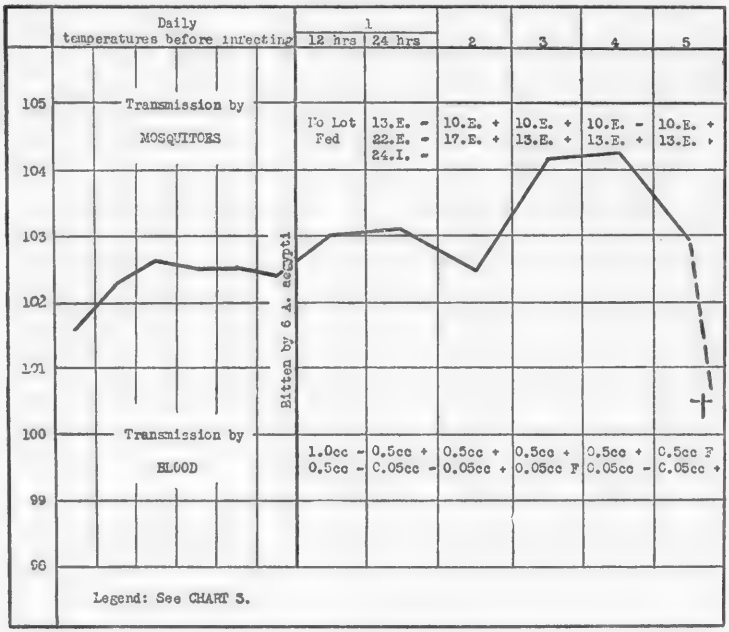


CHART 5. *M. rhesus* E

transmitted, the 10 and 16-day tests, in which 34 and 31 mosquitoes, respectively, were used, being failures. The 48-hour lot induced infection at the 13-day test, but failed at 10 days, the latter animal later proving insusceptible to virulent material. The lots fed on this and the following monkey were manipulated so that by segregation of the insects engorging at the first test-feed, subsequent feeds by previously tested insects were insured.

All blood subinoculations were fatal, employing 1 cc. and 0.5 cc. doses at the end of the first 12 hours and 0.5 cc. and 0.05 cc. at the 24-hour intervals.

*M. rhesus* E was infected by the engorgement of 5 *A. aegypti* and displayed the usual course of experimental yellow fever and typical postmortem findings. On the third and fourth days after infecting, the temperature was the highest (104.3°), preceded by a two-day incubation period and followed by a lowered temperature on the day of death. As with *M. rhesus* D, blood subinoculations were made at the end of the first 12 hours as well as at the 24-hour intervals; batches of mosquitoes were fed, however, only at the regular one-day periods, the last engorgement occurring 9 hours before death.

The results of the tests are recorded in Chart 5, together with the temperatures before and after infecting. All mosquito batches fed on this *rhesus* became infective with the exception of the first-day lot which failed to produce disease either by biting or injection. All tests after incubation periods of 10 or more days in the mosquitoes were successful, excepting the failure to produce disease by the fourth-day lot in an animal later shown to be insusceptible. The fifth-day batch became infected 9 hours before the death of the animal.

Neither dose of blood subinoculated at the end of the first 12 hours induced fatal yellow fever, and 24 hours after infecting, only the larger amount (0.5 cc.) was lethal. Blood drawn the second day transmitted fatal disease in 0.5 cc. and 0.05 cc. On the following 2 days, corresponding to the febrile period, the larger dose was effective. On the last day, 10 hours before death, 0.05 cc. transferred the disease fatally, the monkey that received the larger dose showing a delayed fever and recovering.

## DISCUSSION

In order to interpret the results of these experiments, it is desirable to consider the course of experimental yellow fever in *M. rhesus*. The interval from the time of inoculation until death we have called the course of disease in the experimental rather than in the clinical sense. Clinically, it is difficult to compare the period of symptoms in the monkey with that in man. In the monkey, malaise and loss of appetite do not usually begin until a day or so before death. Vomiting rarely occurs and only when the animal is moribund. To determine the acute disease in *rhesus* monkeys, the urine findings and temperature records are alone available, but the examination of urine is not practical as a matter of routine. As regards temperature, various factors, such as a concurrent infection and excitement due to handling, may alter the reading. However, fairly reliable results are obtainable and records can usually be depended upon when the pre-inoculation temperature, the degree of apparent excitability and the presence of emaciation or diarrhea are taken into consideration.

For practical purposes, under the conditions existing in Nigeria, we have considered as fever a temperature of 104°F. or more, occurring after the expected period of incubation. Upon analysis of the records of 606 monkeys that died of yellow fever, only 3.8 per cent failed to reach this arbitrary limit and 1 per cent showed no registered rise, although it is possible that a brief thermal elevation may have occurred unrecorded. In considering an individual monkey, however, any appreciable rise may be significant.

In the application of these experimental results to the problem of yellow fever in man, the temperature records and the final outcome may profitably be compared. The five monkeys presented courses varying in length and with different thermal curves, but all were within the variations commonly observed in the experimental disease.

It is obvious that the multiplication of the virus in the body of the susceptible animal is extremely rapid, subinoculations of blood at the end of the first day regularly causing fatal disease in other monkeys, the same results being obtained at the end of the first 12 hours in the only blood-infected monkey tested at so short an interval. By the use of even a more delicate intermediary, the mosquito, the virus was transferred from mosquito-infected monkeys by bite at the end of the second day and from blood-infected animals as early as 24 hours after infecting. Moreover, high fever was induced in a test animal injected with insects fed on *M. rhesus* D 12 hours after its inoculation with blood-virus; although it recovered, a subinoculation during this fever resulted in fatal yellow fever in another *rhesus*. In considering these results in relation to the onset of fever, the virus proved by blood subinoculations to be in the circulating blood one and two days before fever. Of even greater interest is the fact that the virus was sufficiently highly concentrated for *A. aegypti* to become infective in every case on the day before the febrile onset.

In view of this early massive invasion of the animal host by virus, it is not surprising that the blood infectivity should continue through the febrile period. This was found to be true when infection was caused by mosquitoes as well as by blood injections. The two monkeys that received the smaller amounts of blood from *M. rhesus* E during its fever are the only recoveries from tests during this period; since mosquitoes coincidentally fed became infective, these failures

were probably due to incidental relative insusceptibility of the injected animals.

The thermal curves varied among the *rhesus* after the primary febrile period and are comparable to the variation seen among human cases at the corresponding interval. In the rapidly fatal course of *M. rhesus* D, the virus persisted in high concentration and was carried by blood and mosquitoes up to 10 hours before death, which was the final period of test. The same results were obtained from *M. rhesus* E, even though death occurred 36 hours after the last registered fever, except that only the final smaller dose of blood (0.05 cc.) was fatal. In the other instances, death occurred on the sixth, eighth and tenth days and was preceded by terminal rises in temperature. The mosquitoes did not become infective from these animals during the latter days and blood subinoculations were variable in result. It appears that in courses of longer duration, the virus tends to disappear from the circulating blood before death.

It should be noted that in the case of *M. rhesus* C, death of the animals did not result from the subinoculations of blood drawn the day before the terminal elevation in temperature, but on the two following days, blood caused fatal attacks in three monkeys. This is the only indication in these experiments of a possible reinvasion of the host by the virus coincident with a terminal fever. Of interest also is the fact that in three of the five monkeys on the last day of infectivity, only the smaller of the two doses used produced fatal infection. This occurred with material obtained in one case at necropsy, in another 9 hours before death and in a third instance 29 hours antemortem. Two explanations suggest themselves: either by chance the monkeys receiving the larger dose were relatively less susceptible, or at injection substances were transferred that protected the monkey or locally were inimical to the contained virus before the absorption of the larger amount of inoculum.*

* Hindle in a paper that has just come to hand (*Trans. Royal Soc. Trop. Med. and Hyg.*, 1929, **22**, 405) makes the suggestion that "—when large quantities of infective blood are used, the injection of the serum containing immune bodies at the same time as the virus may help the animal to recover from infection." We may also refer to two *rhesus* in these experiments injected with blood taken on the ninth day from the monkey that had been inoculated with

The results presented here from animal experimentation agree with those obtained by the study of human yellow fever in that the blood was found infectious and the virus transferable by mosquitoes for at least three days within the febrile period, except from *M. rhesus* D which died after the short interval of 82 hours after inoculation. They show also that infectivity for mosquitoes lasted longer than the initial rise in temperature and in two instances persisted to within a few hours of death.

Of more importance is the fact, demonstrated by these experiments, that even before the onset of fever, the virus was of sufficient concentration to infect mosquitoes. *M. rhesus* has proved to duplicate the fundamental features (clinical, pathological and biochemical) of the human form of disease and there is reason to believe that the nature of the virus invasion is the same in man and monkey. If this is true, the results here obtained point to a better understanding of the condition in man and indicate a rational basis of study of some phases of human yellow fever. A close inquiry into the movements of a human case before onset of illness is suggested in order that his quarters might be freed of mosquitoes and the persons exposed detected and possibly treated with convalescent serum as a prophylactic measure (Bauer and Hudson (4)). Such precautions could be further extended to the careful observation and screening of previously exposed individuals. West African natives have yellow fever in variable degrees of severity, from mild, almost unrecognizable attacks to less often severe and fatal illnesses. This being the case, malaise and prostration are less commonly met than in Western Hemisphere subjects and an African native may be up and about while having a fever. Applying to these conditions the evidence furnished by the experiments reported here, the African patient is probably a source of infection for mosquitoes for one or more days before coming under the observation of those in charge of control work.

It has been hoped that immune serum would be effective therapeutically in human yellow fever. If this proves not to be the case,

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the first lot of mosquitoes fed on *M. rhesus* D (Chart 4). After exhibiting prolonged incubation periods, the animal that had received 0.2 cc. blood succumbed to yellow fever, while the other injected with 1 cc. had a febrile attack and recovered.

the explanation may be found in the fact that the virus appears to multiply with great rapidity, and invasion is early complete in the blood and perhaps in the tissues as well. Furthermore, in the study of material from monkeys killed on the first day of fever, we have regularly found, as has been reported by Aragão (6), that there is tissue damage in the form of marked fatty degeneration of the liver and sometimes of the heart musculature and beginning necrobiosis of hepatic parenchymal cells. Occasionally, also, albumin and casts are present in urine on the first day of fever, in the monkey as well as in man.

It has long been supposed that human necropsy material is non-infectious and we have met no record of fatalities attributable to postmortem examinations of yellow fever cases. Aragão (6) obtained negative results by injection of monkeys with necropsy blood and tissue emulsions. These observations point to a difference in the persistence of the virus in man and monkey since *rhesus* postmortem blood and tissues are often infectious.

By these tests we have attempted to gain information on the nature and activity of the virus. The fact that the blood was fatally infectious as early as 12 hours after infecting up to death indicates simple multiplication of blood-virus rather than phases of development in the sense of metamorphosis. On the other hand, it does not explain the period of incubation necessary for the mosquito to become infective, whether by a cycle of development, multiplication, or penetration to the cephalic region. Bauer and Hudson (8) have shown by the injection of macerated mosquitoes that the virus is in an infective state throughout the incubation period in the insect. If, as seems unlikely, there is a change in morphology of either the mosquito-virus or blood-virus, each stage is infectious and the pathology and course of disease induced are the same as obtained under the usual conditions of virus transfer.

In connection with mosquito transmission, some features seem worthy of particular mention. Test feedings of lots of mosquitoes originally fed on monkeys A and B were made after varying periods of incubation, in several cases owing to rapid enfeeblement among the small number of insects. Particularly in tests of lots from *M. rhesus* B were there suggestions that the shortness of the incubation period

might be roughly proportional to the virus concentration in the donor animal. Those insects fed during the 2 days of fever transmitted the virus after 12 and 10 days, respectively, whereas the mosquitoes fed the days before and after fever failed at 13 and 10 days; and the latter lots tested on the same monkeys later induced fatal yellow fever. This idea seemed corroborated when studied systematically throughout the infective period of the course in *M. rhesus* C by test-feeding after 10 and 13 days incubation. The only lot transmitting at the shorter period was that fed at the initiation of fever on the third day after infecting the donor animal. The reasons for the failures of the infective lots originally fed on the second and sixth days are not apparent but it seems probable that only a small proportion of the mosquitoes were able to acquire an infection because of the low concentration of the virus at these times.

The rapidity of the courses in monkeys D and E did not allow for much illumination on these points. While insects fed at the height of registered fever in the former were the only ones transmitting the disease at 10 days, all but one of the infective lots fed on *M. rhesus* E transmitted at this period. It is remarkable that all infective lots fed on this animal, with the one exception, fatally transmitted the disease after 10 days irrespective of the time of original feeding. It is possible that feeding these lots after even shorter periods might have shown the same difference in rapidity to become infective as demonstrated in the previous experiments, especially since the mean atmospheric temperature (85.3°F.) was 10 degrees higher during the incubation periods than that for the first two series and 5 degrees above the mean during the incubations of the lots fed on *M. rhesus* C.

The failures of transmission by lots fed on the fourth day on monkeys C and E at the 10-day tests (see Charts 3 and 5), and on the first and second days on *M. rhesus* D at the 10 and 16-day tests, respectively (see Chart 4), are explained by the insusceptibility of the tested monkeys, as shown by later resistance to known virulent material. It should be noted here that all tests in these laboratories to date, using infected *A. aegypti* after 9, 10 and 11, as well as the accepted 12 days of incubation, have been positive in a total of 20 susceptible *rhesus* when the insects were originally fed at least during the febrile period.

The scope of the present study could hardly include more than a brief series of tests regarding the relation of the incubation in the insect host to the virus content of the donor animals. Evidence in three of the five groups of mosquito lots studied appears to indicate that when fed before or after the febrile period, there is not only a reduced proportion of infective mosquitoes of each lot, but an increased period required for the insects to become infective by bite. It is probable that the incubation periods in the insect host will be found to be influenced by temperature, but variations in transmission among the lots *of any one* series in the present experiments are not attributable to this factor. The greatest difference in the temperature means computed on a 2-hourly basis for the various lots was only 1.5 degrees.

Certain quantitative considerations in regard to the virus are brought out in several phases of these experiments. The animals injected with blood-virus showed a more rapidly fatal course here than did those infected by the bite of mosquitoes—a fact which has been observed in a large number of other tests. Similarly, blood subinoculations transferred the virus earlier and later in the course of disease, than did the insect host. The monkeys subinjected with blood died after varying intervals, but upon analysis it was found that in general the number of days before death depended upon the stage of the disease of the donor animal. The shortest average intervals (5.3 days) was shown by the 20 monkeys injected with blood of the febrile period, the next longer interval (6.5 days) by the 14 that received blood of the incubation period, and the longest (8 days) by the 16 inoculated with post-febrile blood. We have not found in other experiments that lethal doses of blood induce intervals before death depending in length on the size of the inoculum, except rarely when the minimal lethal dose is approached. When the result of the larger dose was compared with that of the smaller dose in these tests, the average intervals before death were the same, 6 days, in 27 and 23 monkeys in each group, respectively. We conclude, therefore, that quantitative factors did not control the time-result of injections of blood drawn during the periods of disease. It appears, rather, that the results depended on the "activity" of the virus or its ability to establish itself in the subinoculated animals, which was contingent on the stage of disease in which the virus was taken. The same considerations in

connection with mosquito transfers of virus did not reveal any relation between the number of mosquitoes or time of original feeding and the character of the course in the test animals.

These experiments suggest assistance in following certain laboratory procedures connected with the study of yellow fever. For transfer of virus, blood may be drawn with the expectation of a positive result over a wider period than for the transfer by mosquitoes. Small amounts are preferable to massive doses late in the disease. Mosquitoes appear to become infective by feeding throughout the febrile period. The proportion of an insect batch that picks up the virus at other times is probably low. We have not compared the titration of blood-virus and tissue-virus throughout the course of disease, but if the height of virus content of tissues corresponds with that of the blood, the optimum time for the preparation of tissue vaccine seems to be in the febrile period. If serum-virus is used for making vaccine, which has been found experimentally effective (Klotz and Hudson, unpublished), blood drawn during fever is undoubtedly desirable.

## SUMMARY

Five *M. rhesus* fatally infected with yellow fever virus ran varied and typical courses, death occurring from 82 hours to 10 days after infecting. Batches of *A. aegypti* were fed daily on each monkey and specimens of blood injected into other animals. By mosquito transfer, the virus was found to be circulating in the peripheral blood 1 or 2 days after the infecting and the same interval before the onset of fever; in one instance, mosquitoes became infectious by feeding on a monkey 12 hours after its inoculation. Mosquitoes continued to acquire infectivity during the febrile period and for 1 day thereafter, except in one instance when death occurred during fever which prevented post-febrile testing. By subinoculation of blood, the disease was transferred before and after, as well as during the same interval as in mosquito transmission. In one of two attempts, the virus was carried by this means as early as 12 hours after the donor animal was infected. Following the first day of the post-febrile period, blood transmissions were irregularly fatal beyond the period infective for mosquitoes.

These results point to a remarkably rapid multiplication of the virus

in the animal host, in one case a blood subinoculation (0.5 cc.) being successful at the first test 24 hours after the donor monkey was bitten by only 2 *A. aegypti*. The regular acquisition of infectivity by mosquitoes fed during the incubation period is of especial interest in indicating the infectivity of human cases for mosquitoes before the appearance of clinical symptoms. This offers one explanation for the insidious propagation of epidemics of yellow fever and should be useful in the institution of control activities during an outbreak of this disease.

We wish to express our gratitude to Dr. Henry Beeuwkes, Director, for giving us helpful suggestions and for placing at our disposal all materials necessary for these experiments.

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# A MECHANISM OF CONSERVATION IN THE KIDNEYS OF THE WINTER FROG*

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A well known peculiarity of the kidneys of "winter frogs" has been recently emphasized by Höber (1) in his studies of renal function by the method of perfusion. During the months of October, November, December, and January, he finds it often difficult to obtain a proper response by the kidney. This is most noticeable in the excretion of water, some kidneys forming no urine at all in spite of copious and long continued perfusion of both the glomerular and tubular circulations. The only explanation of this phenomonon we have been able to find that is based on experimental data, is that offered by De Haan and Bakker (2). They have observed that winter frogs show a definite decrease in their ability to excrete dyes. Fluorescene, a readily diffusible dye which is ordinarily excreted completely in one day, is not eliminated by the winter frog in several days. In the summer frog this substance is found in the urine in a concentration two or three times that of the blood, while in the winter animal the concentration in the urine is often less than that of the blood. Trypan blue, a less diffusible dye, is found in the urine only in a very dilute state. These observations are interpreted as being due to an increased density of the glomerular membrane which prevents their filtration and consequent appearance in the urine.

In the course of a study on experimental nephritis in frogs made by the method of perfusion, we have come in contact with this problem and as certain of our experiments would seem to offer another explanation of these peculiarities in the behavior of the winter kidney, they are here reported.

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## *Methods*

All of the experiments described were done during the months of December, January, and February. The frogs, R. catesbiana, averaging 850 grams in weight, were obtained from Louisiana. Here the animals hibernate intermittantly from about December 1st to April 1st, as during this period two or three light freezes and several frosts may occur. These cold spells were particularly severe during this last winter, (1928–1929) and it was on frogs collected in this period that our experiments were done. On arrival at the laboratory, the frogs were kept in large tanks of running water at a temperature of about 15°C. and were used within a week or ten days. Under these conditions they were quite active, and had all the appearance of summer frogs.

The method of perfusion which we have used is similar to that of Höber with the important modification of the perfusion pressures, our arterial pressure equaling 45 centimeters of water, while the venous pressure was 20 centimeters. The importance of this modification we have discussed in a previous paper, and will mention again in conclusion.

The perfusion fluid was the modified Locke's solution of Barkan, Broemser and Hahn (3) buffered to a pH of 7.4 and properly oxygenated. Glycocol to a concentration of .50 percent, and glucose to .05 percent were also added to the solution.

The details of the operative procedure have been previously described and will therefore not be repeated. Canulae from the perfusion flasks were placed in the anterior abdominal vein, thus supplying the renal-portal system, and into the aorta just below the junction of the two thoracic branches. Uretral cannulae were placed in the ureters, and the urine collected in measured periods of time. The samples of urine obtained were expressed as to volume in cubic centimeters per hour, the presence or absence of sugar tested with Benedict's Solution, the electrolyte content determined with a Christiansen ionometer, the reading being expressed as a percent concentration of NaCl, and any dye present determined quantitatively with a Dubosq colometer. All these determinations may be made with considerable accuracy, as the samples obtained are large, the normal volume of urine being from 8 to 10 centimeters per hour.

## EXPERIMENTAL

Before detailing the results of perfusion of the kidneys of winter frogs, it is necessary to describe those obtained by similar procedures with the frog in summer when the organs of the animal are in a state of maximum activity.

Under these conditions, with the method described above the volume of urine from the two kidneys varies, as stated above, from 4 to 10 cubic centimeters per hour.

Sugar is normally absent from the urine, though it may be present in the first fifteen minute sample collected. Any persistent appearance of it indicates, as Höber has shown, a damaged kidney, and is usually accompanied by an abnormal increase in the amount of urine.

The electrolyte content of the urine under normal conditions is considerably lower than that of the perfusion fluid. The lowest figures which we have obtained are 20 percent of that of the Locke's solution, while as an upper limit, we have taken 50 percent. If it approaches nearer than this figure to the salt content of the perfusion fluid, sugar is usually present and the volume is large; in other words, there is evidence of what Höber has shown must be interpreted as tubular damage.

In a previous article (4) it was shown that the two dyes, neutral red and phenol red, are excreted in different manners. The former is eliminated almost entirely through the tubules, while the phenol red is excreted in by far the greater part through the glomeruli.

These two dyes were used in the perfusion fluid at a concentration of 12.5 mg. per 1000 cubic centimeters for neutral red, and 20 mg. per 1000 cubic centimeters for phenol red. The rate of excretion of phenol red by the normal kidney under these conditions varies from .5 to 1.2 mg. per hour. In spite of this variation in rate between the kidneys of different animals, abnormalities can be readily recognized as a rule by the inconstancy of the findings. An abnormal low excretion decreases progressively as the perfusion proceeds to a very low figure and is associated with other evidences of kidney damage, such as a high salt content, or the presence of sugar.

The concentration of the dye in the urine as compared to that of the perfusion fluid (Höber's "concentration factor") varies greatly, affected as it is by the volume of water excreted. A concentration factor as low as 200 percent may be found in urine which is otherwise normal, the rate of excretion of the dye remaining within normal limits due to a large volume of urine. With small volumes of urine as high a factor as 3000 percent may be obtained. On the other hand, when this figure falls as low as 100 percent, the urine contains sugar, a high sa t content, or some other evidences of kidney damage are present.

The rate of excretion of neutral red also varies considerably with different kidneys. From .45 mg. to 1.5 mg. per hour have been found under normal conditions, but here again the constance of a normal kidney is easily distinguished from the progressive fall observed with abnormal organs. The concentration factor, as in the case of phenol red, is dependent largely on the rate of water excretion. A factor as high as 7500 percent has been observed.

### *The Perfusion of Normal "Winter Frog" Kidneys*

In the first five periods of Figure 1 is shown a typical experiment illustrating the effect of perfusion on the kidney of the winter frog.

The dye in the Locke's fluid was phenol red. It will be noticed that although in the first period the volume of water was within normal limits for summer conditions, 9 cubic centimeters per hour, during the first five periods there is a gradual and progressive decrease to 2.4 cubic centimeters per hour. The arterial perfusion flow was practically constant during this period, so that the decrease in volume can not be explained by any failure of the perfusion method. A faint trace of sugar was present in the first period, but disappeared in the next sample and remained absent during the remainder of the experiment. The salt content in the first period was 50 percent, decreasing to 45, and finally to 40 percent of the concentration in the perfusion fluid in period 5.

The phenol red, as in most of the experiments to follow, was introduced first into the tubular circulation, and then into the glomerular system. This was to test our previous findings, that the excretion of this dye is chiefly through the glomerulus. As is seen in periods 1 and 2, only a very low rate of excretion resulted, .02 and .04 mg. per hour. The rate rose however at once to .16 mg. per hour when it was led to the glomeruli, an eight fold increase. This rate is, however, still far below the usual figure for a summer frog, and in the following periods 4 and 5 it decreased progressively to a final figure of .07 mg. per hour. The highest concentration factor attained was only 115 percent.

The experiment described thus far, differs strikingly from the results of perfusion of a summer frog in that there was a gradual and progressive decrease in the excretion of all the elements of the urine. The water decreased, sugar disappeared, and salt became less, as did also the rate of excretion of phenol red. The concentration factor of the latter was, however, greater than 100 percent. In other experiments similar results were obtained, in some the volume of urine decreasing to less than .1 cubic centimeter per hour, and in other cases the volume from the beginning of the perfusion was so low that the perfusion might be said to be ineffective.

Viewing these findings in the light of De Haan and Bakker's theory that the winter frog's kidney is characterized by a decreased filtration of the urinary constituents through the relatively impermeable glomerular membrane, it is difficult to explain by such a mechanism

the concentration of the dye in the urine above that of the perfusion fluid. In other experiments, this concentration factor of phenol red rose to such figures as 410, 520, 540, 840, and even 1000 percent as the volume of water decreased. Obviously the concentration mechanism is still active in the winter frog's kidney and this fact led to the idea that perhaps all of its peculiarities might be explained by the assumption of an increased absorptive activity on part of its tubules.

The hypothesis is easily tested. The absorptive function of the tubules may be depressed, as Höber has shown by anesthesia with

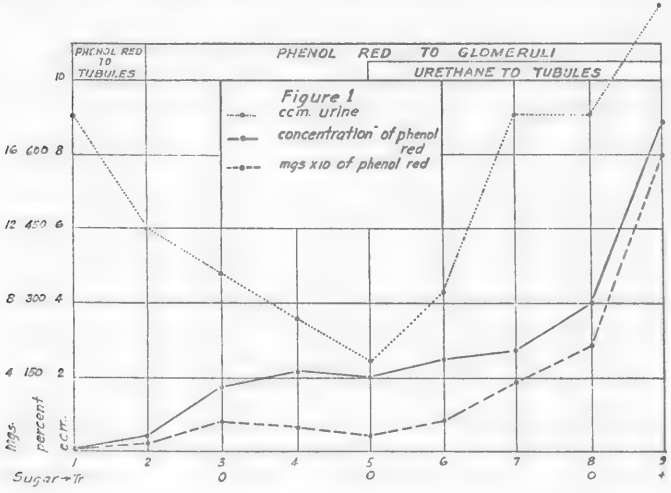


FIG. 1

urethane, and if our assumption is true, such a procedure should cause the kidney of the winter frog to form a urine which is similar in amount and constituents to that of the summer frog. The second half of figure 1 (periods 6, 7, 8, and 9) shows such an experiment.

Beginning at the removal of sample 5, urethane in a concentration of 2 percent was added to the tubular perfusion fluid, and in periods 6 to 9 all the evidences of a depression of tubular function as described by Höber and observed by us in our previous article developed.

The rate of water excretion increased steadily until it reached 12 centimeters per hour, a figure which equals the output of the summer frog. An equally striking increase is noted in the output of phenol red. The rate of excretion was

doubled in the first period following the anesthesia and progressively increased until in the ninth period it reached a figure which equals even the maximum figure attained by summer frogs, that is, 1.6 mg. per hour.

That these increases in water and dye output are associated with a failure of tubular absorption, is seen by the changes in salt and sugar excretion. Absorption of salts remained active until the eighth period, when the concentration rose to 50 percent of that of the perfusion fluid concentration and finally to 60 percent in the last period, at which time a trace of sugar also appeared in the urine.

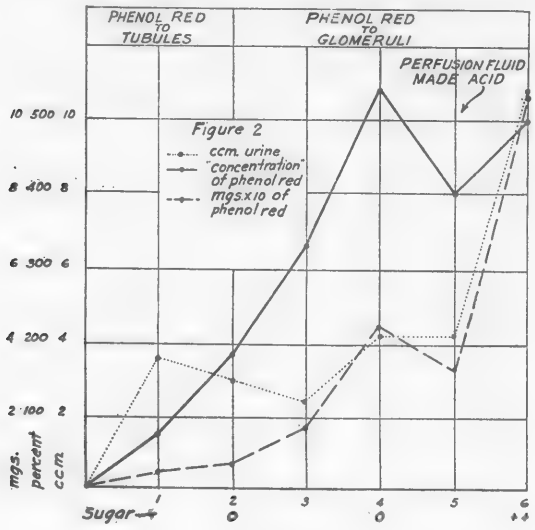


FIG. 2

These results are not the peculiar effect of the anesthetic, for any damage to the tubular epithelium which produces a depression of its function results in similar effects. In figure 2 the depression of tubular absorption was produced by frank damage to the epithelium with an unsuitable acid perfusion fluid. Under these conditions the scanty winter urine, poor in dye content, not only increased to values in water and dye which might be considered normal for a summer frog, but continued to show a decrease in tubular absorption until a point of frank abnormality was reached.

The perfusion was started with Locke's solution at the normal pH of 7.4 and the usual amount of phenol red. The volumes of urine in the first 5 periods were

low or moderate in amount, and contained no sugar. While the phenol red was administered to the tubules, (periods 1 and 2) the rate of excretion was very low, .08 mg. per hour, and increased over five fold to .44 mg. per hour, when it passed to the glomeruli (period 4). This figure is however lower than that of the average summer frog and begins to fall in the 5th period. At this time the perfusion fluid to the tubules was made acid to a pH of 5.9. The epithelial cells were immediately damaged, and their absorptive activity destroyed as evidenced by the appearance of sugar in the urine. As a result, the rate of water output rose to what might be considered a normal figure for the summer frog, 10.8 cubic centimeters per hour, and the rate of excretion of phenol red to the high rate of 1.6 mg. per hour.

These experiments support strongly the idea that the apparent "inactivity" of the winter frog's kidney is the result of an increased activity on the part of its absorbing tubules rather than that there is any decrease in the process of glomerular filtration. Both water and dye are present in sufficient amount in the tubular lumen and are excreted readily when the tubule cells are prevented from absorbing them.

In considering the latter part of the two experiments just described, in which the excessive absorbing activity of the tubules has been depressed either by anesthesia or by frank damage to the tubule cells, one is struck by the fact that not only is a large amount of dye excreted, but also that although the normal concentrating mechanism, absorption of water, has been depressed, never the less the dye is still found in considerably higher concentration in the urine than in the perfusion fluid. Evidently then, some water is being absorbed from the glomerular filtrate or there is an added source of dye output in these periods, or both of these possibilities exist.

Although it is impossible to answer such an involved question in any completely satisfactory quantitative manner, it can at least be shown that the second named factor may well play an important part in the concentrating process. The following experiment (Fig. 3) shows that under certain conditions an added source and mechanism exists for the excretion of phenol red which may not function to any significant degree under normal conditions of renal activity.

Clear Locke's solution was supplied to the tubules while phenol red in the usual concentration was introduced into the glomerular circulation. The dye was excreted in high concentration, (780 percent) and at the moderately high rate of

.56 mg. per hour. As is typical of the winter frog, the next period showed a drop to about one half this figure, .25 mg. per hour, the concentration factor was lower and the volume of urine decreased. At the end of this period the dye containing fluid of the glomerular circulation was replaced with clear Locke's solution, so that no dye was being supplied to the kidney. Nevertheless, the rate of dye excretion remained practically constant, .22 mg. per hour and since the volume of water had continued to decrease, the concentration factor was somewhat higher, 620 percent. The following period also showed a high rate of excretion, but in the 5th and 6th periods the lack of dye supply began to make itself apparent, and the rate of excretion and the concentration factor of the dye fell to a low figure.

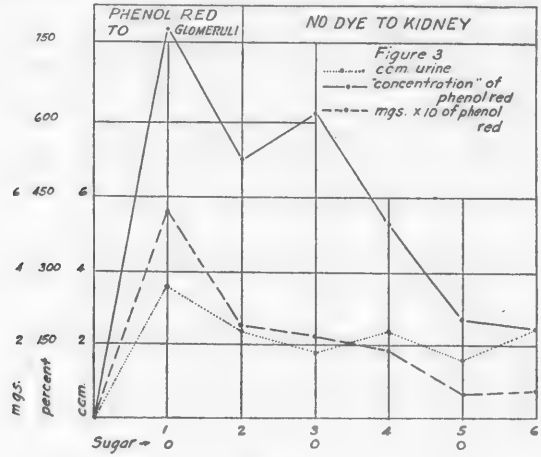


FIG. 3

For one half hour, therefore, the kidneys had maintained the rate of excretion and concentration of the dye without the usual source of glomerular supply. Since no other tissues were included in the perfusion system, it is obvious that the source of this excreted dye must have been the kidney itself.

It has been shown by many observers that a storage and concentration of phenol red occurs in the cells of the renal tubule (Marshall and Crane (5)) and Hayman and Richards (6) have proved by direct observation that when introduced into the capsular space of the glomerulus the dye appears within the tubule cells. Richards and Barnwell have furthermore shown (7) that although normal tubule cells are able to maintain a higher concentration of phenol red than

exists in the fluid in which they are immersed, when damaged this is no longer the case. With these facts in mind, it seems clear that the dye which had been stored during the first periods of the experiments described was subsequently liberated from the tubule cells and excreted during the period of dye free perfusion. Such a source and method of dye excretion is particularly important in the winter frog, for the kidneys of such animals whose absorbing mechanisms are extraordinarily active, are especially rich in stored dye, so that when damaged by the urethane as in experiment 1, a considerable amount may be added by liberation from the tubule cells to that amount which is being eliminated by glomerular filtration.

Evidence has been presented which has been interpreted as indicating that the kidneys of winter frogs conserve water, salt, sugar, and, when present in the urine, dye, by a process of excessive absorption from the urine within the renal tubule. It has been shown by Schminzky (8) and by the writers, that neutral red is excreted almost entirely through the tubule epithelium. Its manner of excretion is therefore different from that which we have found for the excretion of phenol red. In the kidneys of the winter frogs which are so active in conservation by their mechanism of absorption, one m'ght expect a similarly conservative action on the part of the excretory function of the tubules. The following experiment shows this to be the case (Fig. 4).

Neutral red was introduced into the tubular circulation. In the first period it was excreted at a moderate rate (.45 mg. per hour) and in high concentration (735 percent), but in the next two periods (2 and 3) there was a marked fall in both rate of excretion and concentration. The final figure of .03 mg. per hour and a concentration factor of 100 percent are far below any figures obtained with normal summer frogs. The volume of urine decreased and the absorption of salt was active as its concentration in the urine was only 40 percent of that of the perfusion fluid in the 3rd period.

The kidneys of the winter frog therefore excrete neutral red to a lesser degree than those of the summer frog and the conservative mechanism of excessive absorption is complemented by a decrease in the excretory function of the tubular epithelium. The experiment was now continued for the examination of another point of interest.

It has been shown that phenol red which has been absorbed by the tubule cells from the lumen of the tubule may be subsequently excreted back into the lumen when the concentration of dye in the urine is low or when the tubule cells have lost their absorptive power as a result of damage.

The findings in the fourth and fifth periods show that there may be an analogous excretion of stored neutral red from the tubule cells. At the end of period 3 the perfusion fluid containing neutral red was replaced by dye free Locke's solution. The kidney was now receiving no dye, yet during the next period the rate of elimination of neutral red remained nearly as high as the preceding period and the urine was more concentrated in dye than the perfusion fluid. By the sixth period both rate of excretion and concentration factor had fallen, evidently due to the exhaustion of the available neutral red which had been stored in tubule cells.[1]

It will be noticed in the sixth period that the volume of urine was small, 1.2 centimeters per hour, and the salt concentration was especially low, only 30 percent of the concentration of salt in the perfusion fluid. This is strong presumptive evidence that we are dealing with typical winter kidneys, that is, that their absorptive mechanisms are very active. Nevertheless, this point was tested with phenol red as in our previous experiments. This dye was supplied to the glomeruli during the 7th period and in the 8th period was excreted at a moderate rate, .24 mg. per hour and in moderately high concentration, 495 percent. At the beginning of the 9th period tubular absorption was depressed by administration of urethane in a concentration of 2 percent to the tubules. The rate of water elimination immediately rose to 10.6 centimeters per hour and there was an increase in the rate of phenol red excretion to .66 mg. per hour. The absorptive mechanism of the tubules had indeed been so depressed by the anesthetic that the concentration of salt rose to 60 percent of that of the perfusion fluid and sugar appeared in the urine.

In the same winter kidneys, therefore, an excessive absorptive activity and a decreased excretory function was demonstrated in the tubular epithelium.

## DISCUSSION

From the experiments described above, the conclusion has been drawn that in winter frogs there exists a profound alteration in the function of the tubular apparatus of the kidneys. This alteration is manifested by an increase in the activity of their resorptive processes and a decrease in their excretory functions. The method of demon-

[1] Scheminzky has described the storage of neutral red in the tubular epithelium and we shall later discuss this point more fully in a study of experimental nephritis by means of vital staining.

stration of these facts included the study of substances such as water, salts and sugar, which are normally present in the blood and urine, but the most striking demonstration was made with the dye, phenol red.

In a previous article (4) we have shown why we believe that the principal method of phenol red excretion is through the glomerulus.

While this article was in press, there appeared a study by Scheminzky, working in Höber's laboratory, who from somewhat similar experiments came to a different conclusion. He found, as did we, that neutral red was excreted by the tubules and only slightly by the glomerulus, but contrary to our conclusions, he believes that phenol red is also excreted principally by the tubules.

It is impossible to adequately discuss this important and lengthy paper, and we shall only attempt to call the reader's attention to certain points which we feel should be especially noted in comparing the results of the two investigations.

In the first place the type of experimental animal is different. Scheminzky used winter animals, and such material judged in the light of De Haan and our present paper is obviously not the most satisfactory for the study of the method of normal dye excretion. As a result, the volumes of urine, and amounts of dye excreted are very small. In one emphasized experiment .19 cubic centimeters per kidney per hour is given as a normal volume and in another .86 cubic centimeters is cited as an example of diuresis due to tubular narcosis. The technical difficulties of quantitative NaCl and phenol red and qualitative sugar determinations all on a thirty minute sample under these conditions are manifest, especially when dye output must be expressed in millionths of grams.

Of even greater importance, however, is the difference in perfusion technique in Scheminzky's and our study. In most of his experiments he used a pressure of 18 and 9 centimeters of water for the arterial and venous systems and under these conditions the venous flow was greater than the arterial.[2] In our previous article

[2] In discussing the improbability that fluid from the venous circulation has reached the glomeruli Scheminzky on page 667 gives a theoretical calculation as evidence of the impossibility of such an occurrence. Without going into the details which he presents we wish to point out that although it is correct that at least 9.1 centimeters of dye containing perfusion fluid must be assumed to have reached the glomeruli from the venous circulation in his hypothetical case, it is not at all necessary to assume that this was mixed in the glomeruli with the 40 cubic centimeters of dye free fluid which flowed through the aortic circulation. The flow through the glomerular capillaries may in fact have been purely venous and

we have pointed out that in order to cause the kidney to function in a normal manner pressures of 45 and 20 centimeters are required. Detailed experiments have shown that such pressures produce no abnormalities in the formation of the urine by either the glomeruli or tubules and that only with them will perfused kidneys secrete a volume of urine which is comparable with what Adolph (9) has shown to be the normal rate of water excretion.[3]

As a result of these seasonal and technical differences, and also because our frogs were perhaps much larger, our volumes of urine are larger and may be considered as normal in regard to water excretion. The determinations of its constituents are made with ease and the results of dye excretion are expressed as milligrams.

So much for general differences in method. In the specific experiments certain differences should also be noted. In contrasting the excretion of phenol red by the tubules and by the glomeruli Scheminzky used different animals to test each part of the renal unit. The dye was found in the urine in concentrations higher than in the perfusion fluid after both tubular and glomerular administration and these results on different animals were then compared by averages and by "specific concentration" and "Leistungs" factors rather than by rates of excretion, for these rates varied in different animals. In our experiments the comparison of the excretion of either dye by the two parts of the kidney, glomerulus and tubule, was done in consecutive periods of a single experiment in the same animal and the contrasting results expressed as simple rates, i.e., mgs. per hour.

In regard to the effect of narcosis of the tubules on the excretion of phenol red, Scheminzky states that in five of twelve experiments although the anesthetic produced its typical effect on all the other urinary constituents, it did not depress the rate of excretion of the dye, as is required to support his hypothesis of tubular secretion. Doses of from two to five times the size which produced these results were required to regularly produce a depression of dye elimination. This is ex-

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in amount equal to any fraction of the total venous flow, the arterial fluid being shunted around the glomerular tuft by way of the arteriae rectae to join the intertubular capillaries. Such anastomoses have been shown by Nussbaum and Hayman to mention only the first and last of a long series of investigators. Under these conditions, which are as proper an assumption as that of Scheminzky, it would be a simple matter to account for the total amount of dye found in the urine as a result of venous rather than arterial perfusion of the glomerular tuft.

[3] As a matter of fact the pressures used by us are even less than the maxima that have been observed existing in the living frog's circulation by many other observers. Hayman gives a figure of 60 centimeters of $H_2O$ as a possible normal aortic pressure, the average being 37 centimeters. Our venous pressure could doubtless be reduced, and this would lessen the danger of a contaminating venous perfusion of the glomerulus, but we have selected the pressure given above as proper to insure a complete circulation around the tubules of the entire kidney.

plained by the assumption that the secretory function of the tubule is more resistant to narcosis than the absorptive process.

Another explanation is, however, possible and that is that the larger doses of anesthetic affect more of the renal apparatus than the tubule. In a series of experiments on experimental nephritis in the frog to be published later, in which attempts were made to cause selective damage to various parts of the kidney, great difficulty was encountered in damaging any one part of the renal unit without affecting to some degree the other elements. The glomeruli, the tubules, and especially the vascular system are all susceptible to damage when strong toxic agents are administered even with great care to any one portion of the kidney, and under these conditions of wide spread damage its function is altered in such a complex way that it is impossible to judge as to the part played by any single structure in the production of the total effect.

Our experiments on the winter frogs have also shown an accessory mechanism for the elimination of phenol red by the kidney. As the following table shows, although the increases after tubular damage in rates of water and dye excretion are in most cases of similar magnitude, occasionally the dye elimination is increased to a far greater degree.

| Increase in water elimination | Increase in dye excretion |
|---|---|
| 2.5 | 2.7 |
| 8.2 | 7.3 |
| 2.3 | 2.2 |
| 2.5 | 3.2 |
| 5. | 22.0 |

The last figure of this group, which is from an experiment in which there was a long period of dye absorption before the administration of the anesthetic to the tubules, it would appear that there is some other source of dye than that responsible for water output. As our experiment 3 shows, this added source is doubtless the dye which has been absorbed from the lumen and stored in the tubule cells. An "anomalous secretion" of such stored dye is apparently possible under unusual conditions, such as, for example, when the concentration of dye in the lumen of the tubule falls far below that in the tubule cells, or when these cells are damaged and no longer able to hold it in concentrated form. Here again the phenomenon is best observed in winter frogs for their tubule cells are more highly charged with dye as a result of their excessive absorptive activity.

Our experiments on the activity of the winter frog's kidney also add a further demonstration of the difference in the manner in which the kidney handles neutral red and phenol red. In such animals with neutral red the concentration factor is usually low when the rate of excretion has fallen to a low figure. With phenol red, the concentration factor is always greater than 100 percent, and often considerably larger, even though the rate of excretion is small. This difference also may be explained by our previous theory of tubular and glomerular excretion of the two dyes. The method of concentration of neutral red is a single mechanism in that it is a function of the tubule alone,

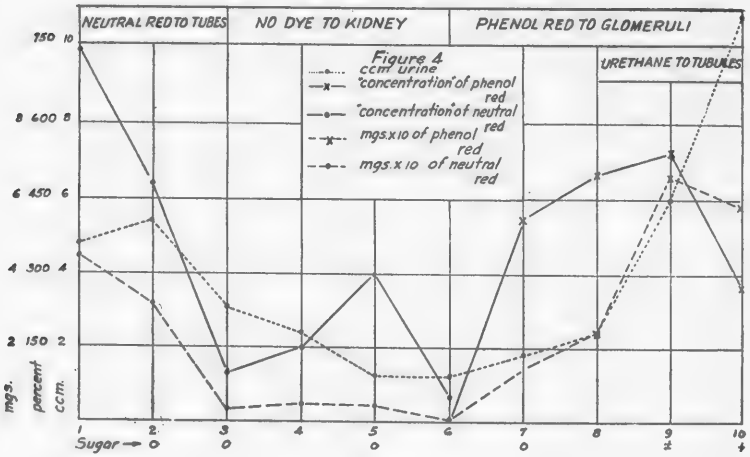


FIG. 4

namely tubular excretion of dye and tubular absorption of water. In the case of phenol red the concentration of the dye is the result of the activity of at least two mechanisms, glomerular excretion and tubular absorption of water and to a lesser degree of dye. An increased activity of the latter may therefore reduce the amount of dye excreted into the final urine without decreasing its concentration, since it is reasonable to suppose that water is absorbed more readily than the dye. The method of "anomalous secretion" of phenol red which we have just described may also aid in producing the result of a high concentration but under these conditions the rate of excretion is apt also to be high.

## CONCLUSIONS

1. A method of conservation has been demonstrated in the kidneys of winter frogs.

2. The mechanism of this conservation is an increase in the absorptive function and a decrease in the excretory activity of the tubular epithelium.

3. The excessive absorptive process may be depressed by various means. Such a depression in the tubular activity is followed by large increases in the rate of excretion of water, salts and, if present in the urine, phenol red.

4. Further evidence is thus obtained which supports the theory that phenol red is excreted chiefly through the glomeruli.

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# BACTERIOLOGY OF THE BLOOD AND JOINTS IN RHEUMATIC FEVER*

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Although thirty years have elapsed since a streptococcus was first reported in the lesions of rheumatic fever, the etiology of this disease still remains in doubt. It is true, of course, that the streptococcus is now looked upon by many as the exciting agent, but final acceptance of the streptococcal theory is far from being universal.

When one seeks for an explanation of this disagreement, the answer is not hard to find. From time to time streptococci have been found both in the blood and in the joints of patients with acute inflammatory rheumatism, but rarely with sufficient frequency to justify the conclusion that they were the cause of the disease. Indeed, several criticisms can fairly be made of previous investigations in this field; the number of cases studied has usually been small; some authors have failed to state the exact percentage of positive cultures obtained; and control cultures have not been made on individuals free from rheumatic fever.

Triboulet and Coyon (1), in 1897, cultivated a diplococcus from five living cases of rheumatic fever, and at autopsy on one fatal case. Later (1898) they (2) described the production of mitral endocarditis in rabbits by the intravenous injection of a diplococcus isolated from the blood of a patient with rheumatic fever. In another article Triboulet and Coyon (3) reported that in 11 consecutive cases of rheumatic fever they had isolated a diplococcus from the bloodstream.

Apert (4) in 1898, using the methods of Triboulet and Coyon, took blood cultures on two cases of chorea. One yielded a diplococcus similar to the strains recovered by these investigators. In 1899, Westphal, Wassermann and Malkoff

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(5) made a bacteriologic study of a fatal case of rheumatic fever with chorea and endocarditis. A streptococcus was recovered from the blood, the brain and the heart valves. Experiments were conducted on 80 rabbits with the production of arthritis in a considerable number.

In 1900, Poynton and Paine (6) demonstrated diplococci in eight cases of acute rheumatic fever. Five of the eight cases, however, were fatal, and in these five the cultures were obtained at the post mortem table. In three of the eight cases blood cultures were taken; two out of the three were positive for diplococci. In the remaining five cases, diplococci were isolated from pericardial fluid, vegetations on the heart valve or from the tonsils. When injected into rabbits these diplococci produced arthritis, valvulitis and pericarditis.

Philipp (7), in 1903, took blood cultures on 31 cases of rheumatic fever and obtained entirely negative results. He concluded that rheumatic fever is a specific infectious disease of unknown etiology.

Loeb (8), in 1908, took blood cultures on 45 cases of rheumatic fever. In eight patients a streptococcus was obtained which morphologically and culturally corresponded to that described by Poynton and Paine (6) and other workers.

In 1908 Beattie (9) examined three cases of fatal rheumatic fever at the post mortem table. In all of them cultures from the heart's blood remained sterile. In all three cases, however, streptococci were grown from pieces of synovial membrane. These strains were identical in their cultural characteristics, and differed in no way from Poynton and Paine's "streptococcus rheumaticus."

In 1913 Rosenow (10) reported the isolation of streptococci from the joints in seven out of eight cases of acute rheumatic fever. The same author (11), in 1914, recovered streptococci from the bloodstream in four out of seven patients with rheumatic fever. Rosenow divided his streptococci into three groups, according to their effect on blood agar: 1. Those producing green; 2. Those producing slight hemolysis; 3. Those producing no perceptible change. Rosenow claimed that under certain conditions any of the three varieties could be converted one into the other. When these streptococci were injected into rabbits, they induced arthritis, endocarditis and pericarditis.

Herry (12), in 1914, undertook an elaborate study of rheumatic fever based on sixty cases. Forty-three out of the sixty cases yielded positive blood cultures, in every case a diplococcus similar to that described by Poynton and Paine. Four out of five joint cultures were positive for streptococci, and seven pleural fluids all yielded streptococci. Altogether 47 of the 60 cases (78.3 per cent) yielded a streptococcus from some one of these sources. Herry found that arthritis, myocarditis and endocarditis were readily produced in rabbits by intravenous injections of these streptococci.

Swift and Kinsella (13), in 1917, cultured the blood from 58 cases of rheumatic fever and obtained streptococci from seven, or 12 per cent, of them. The joints were cultured in 25 cases with entirely negative results. All of Swift and Kinsella's streptococci produced green on blood agar plates, but no strict biological

relationship could be established between these strains by complement-fixation tests. No agglutination tests were made.

In 1925, Clawson (14) isolated streptococci from the blood in a "relatively high percentage of cases of rheumatic fever." His technic for blood cultures differed from that of his predecessors in several respects: 1. Fifty c.c. of blood were collected for culture. 2. Only the clot was used for cultivation. 3. Cultures were observed for one month before being discarded. Altogether twenty strains of streptococci were studied. With one exception they all produced methemoglobin after a period of cultivation. The exceptional strain was a typical streptococcus hemolyticus. By injecting these streptococci into rabbits, Clawson found that lesions similar to those occurring in human rheumatic fever could be produced experimentally.

Zinsser and Yu (15) (1928) reported the results of cultures on two cases of rheumatic fever and two cases of rheumatic carditis. In one rheumatic fever patient, intra-vitam blood cultures revealed both an alpha and a gamma strain of streptococcus. The other rheumatic patient died and at autopsy an alpha streptococcus was recovered from the myocardium and from the pericardial fluid. Both of the patients with carditis died. Cultures from the blood of both these patients were sterile, but post mortem cultures from the spleen in each instance yielded a streptococcus.

Surányi and Forró (16) (1928) took blood cultures on 25 cases of "polyarthritis" and obtained green streptococci in 17, or 68 per cent, of the series. The blood was first hemolyzed in citrate solution and the sediment cultured in broth tubes. In contrast to these positive findings is the recent study of Nye and Seegal (17) (1929) on the bacteriology of the blood in rheumatic fever. Fifty cubic centimeters or more of blood was taken in nearly every instance. Twenty-five cases were cultured in this way with entirely negative results.

Small (18) and Birkhaug (19), whose recent studies (1927) on the etiology of rheumatic fever have attracted considerable attention, have not been particularly interested in the bacteriology of the blood and joints in rheumatic fever, but have investigated the gamma type of streptococcus in relation to its presence in the throats of patients with rheumatic fever. Small's original R1 "streptococcus cardioarthritidis" was isolated from the blood, but most of his subsequent strains were obtained from the throats of rheumatic fever patients. Of Birkhaug's 27 strains of "non-methemoglobin-forming streptococci," only three were isolated from the blood, the remainder from tonsils, feces, etc.

It is evident that investigators have met with varying degrees of success in their attempts to recover streptococci from the blood and joints in rheumatic fever. It is significant, however, that those who have recovered streptococci from rheumatic fever patients have almost invariably recovered the same type of streptococcus, namely the streptococcus viridans. Furthermore, although these streptococci

have presented very much the same appearance morphologically and culturally, the trend of evidence is against their comprising one biological group. In addition to the streptococcus viridans, an indifferent streptococcus similar to that described by Small and by Birkhaug has occasionally been isolated.

### *The Present Study*

The present investigation, initiated in the Spring of 1928, followed as a natural corollary to our study on the bacteriology of the blood and joints in chronic infectious arthritis, the results of which have been recently published (20). If streptococci could be isolated frequently from the blood and from the joints in infectious arthritis, it seemed reasonable to suppose that they could also be found in the blood and joints in rheumatic fever, a disease which, though differing from infectious arthritis in many respects, possesses with it certain points of similarity.

It was decided that only those cases of rheumatic fever that showed fever and definite joint manifestations would be studied. Altogether, sixty such patients have been included in this investigation. In almost every instance cultures were taken during the febrile stage and usually before salicylates had been given. During the Spring of 1928, 29 cases were studied; during the Spring of 1929, 31 cases. Most of the patients in the series were adults,* but through the kindness of Dr. Charles Hendee Smith we also had access to the children's wards at Bellevue Hospital. Unfortunately, many of the rheumatic children showed no active joint manifestations, and could therefore not be included in the series.

### *Methods*

The technical methods employed are submitted in detail:

(*1*) *Broth for Blood Cultures.*—Fresh beef heart free from fat is passed through a meat grinder and weighed. For every 1000 grams of meat one liter of tap water is added. The mixture is allowed to stand in the ice-box over night. In the morning, it is heated to 20 deg. C., and filtered through a flannel bag. It is

* We are very much indebted to Dr. Van Horne Norrie, Dr. John Wyckoff and Dr. Charles Hendee Smith for the privilege of using material from their services in Bellevue Hospital.

then boiled over a low flame for one hour with frequent stirring. After filtering through filter paper, water is added in sufficient quantity to make up for loss by evaporation. One per cent bacto-peptone (Difco standardized) and 0.5 per cent sodium chloride are added. The broth is next placed in the Arnold sterilizer at 100 deg. C. for ten minutes to dissolve the salt and peptone. The pH. is adjusted to 7.8 with 2N sodium hydroxide (Kahlbaum) using a La Motte block comparator with phenol red as indicator. The solution is placed in the Arnold at 100 deg. C. for one hour and again filtered through filter paper. The pH. is again taken while the broth is still hot, and is usually found to have fallen to 7.6. If it should fall below 7.6 a further adjustment of the pH. is necessary followed by another hour in the Arnold. The final pH. of the broth should be 7.6. It is poured into 100 c.c. bottles (50 c.c. to each bottle) and sterilized in the Arnold for 30 minutes at 100 Deg. C. on three successive days. The broth is then incubated for 24 hours to test for sterility.

(2) *Blood Broth for Joint Cultures.*—Broth for joint cultures is prepared in the same way. The broth is then poured into ordinary culture tubes (8 c.c. to each tube) and sterilized in the Arnold for 30 minutes at 100 deg. C. for three successive days. To each tube of broth is now added 0.1 c.c. defibrinated rabbit's blood. The broth is then incubated for 24 hours to test for sterility.

(3) *Preparation of Agar.*—Beef heart free from fat is passed through the meat grinder and weighed. For every 1000 grams of meat two litres of water are added. The mixture is allowed to stand in the ice-box over night. In the morning it is heated to 20 deg. C. and filtered through a flannel bag. It is then boiled over a low flame for one hour with frequent stirring. After filtering through filter paper, water is added in sufficient quantity to make up for loss by evaporation. One per cent bacto-peptone (Difco standardized), 0.5 per cent sodium chloride, and 1.5 per cent bacto-agar (Difco standardized) are added. The mixture is placed in the Arnold at 100 deg. C. for 30 minutes to dissolve the salt, peptone and agar. The pH. is adjusted to 7.8 with 2N sodium hydroxide (Kahlbaum). The solution is placed in the Arnold at 100 deg. C. for one hour. It is then passed through a Sharples centrifuge to remove the sediment. The pH. is again taken while the agar is still hot, and it is usually found to have fallen to 7.6. If it should fall below 7.6, a further adjustment of the pH. is necessary, followed by another hour in the Arnold. The final pH. of the agar should be 7.6. It is poured into test tubes (8 c.c. to each tube), and sterilized in the Arnold for 30 minutes at 100 deg. C. on three successive days, allowing the media between sterilizations to stand at room temperature. Tests for sterility are made by incubation for 24 hours.

(4) *Technic for Blood Cultures.*—The syringes and needles used for taking the cultures are enclosed in glass tubes and sterilized in the dry sterilizer for two hours at 150 deg. C. The skin is prepared with two coats of iodin and one of alcohol. Twenty c.c. or more of blood are taken from a vein in the arm. The blood is divided between two culture tubes, allowed to clot, and put in the ice-box over

TABLE 1

*Résumé of 60 Cases of Acute Rheumatic Fever*
*Cases Studied in 1928*

| Patient | Age | Sex | Previous Attacks | Preceding Sore Throat | No. of Days Ill Before Adm. | Joints* Involved | Cardiac Involvement § | Max. Tem. | W.B.C. | Blood Culture† | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | 1st | 2nd | |
| B-929 | 22 | M | 1 | Yes | 21 | W. K. A. H. S. | Systol. Murmur | 104.2 | | — | | |
| B-932 | 20 | M | 2 | | 14 | S. A. K. | Endocard. | 100.4 | | — | | |
| B-856 | 48 | M | Many | | 21 | A. K. F. | Auricul. Fibrill.; Myocard. | 103.2 | 14,200 | RB-7 | | |
| B-375 | 9 | F | | Yes | 10 | A. K. T. | Endocard. | 104.2 | 11,800 | RB-2 | | Pyelitis. |
| B-377 | 32 | M | 2 | | 21 | A. K. | | 101.0 | 10,800 | RB-37 | | |
| B-373 | 7 | M | | | 3 | K. A. | Endocard. (later) | 103.3 | 11,000 | — | | |
| B-356 | 9 | F | | Yes | 30 | F. K. S. W. | Endocard. | 104.0 | 23,400 | RB-6 | — | Rheumatic nodules over olecranon. |
| B-372 | 11 | M | 1 | | 6 | A. T. | Systol. Murmur | 100.6 | 10,100 | RB-3 | | |
| B-924 | 28 | F | | | 10 | K. S. W. F. A. | | 104.2 | 16,600 | RB-1 | — | Acute nephritis. |
| B-434 | 28 | | 2 | | 6 | K. A. W. H. T. | | 102.8 | 12,850 | — | | |
| B-911 | 18 | F | | Yes | 7 | E. W. S. K. | | 103.4 | 13,900 | — | | |
| B-900 | 31 | F | | | 48 | F. T. S. K. A. | Pericard. | 105.4 | 26,600 | RB-38 | | Fever and signs of pericarditis until death 18 days after admission; no sign of endocarditis. |
| B-923 | 20 | M | 1 | | 14 | S. A. | Endocard. | 101.6 | | — | | |
| B-949 | 37 | M | | | 14 | K. E. | | 102.0 | 16,500 | — | | |
| B-2161 | 29 | M | 1 | Yes | 3 | K. A. S. | Endocard. | 103.0 | 11,400 | — | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B-948 | 24 | M | 1 | | 9 | H. | Peri. Endo. & Myocard. | 104.0 | 11,450 | — | RB-4 (PM) | Died; anatomic diagnosis. (1) Serafibrinous pericarditis. (2) Rheumatic endocarditis. (3) Rheumatic nodules. |
| B-903 | 23 | F | | Yes | 5 | F. K. F. S. | | 103.6 | 15,400 | — | | |
| B-909 | 19 | M | | Yes | 9 | K. W. E. | Systol. Murmur | 104.0 | 12,000 | — | | |
| B-917 | 21 | M | | | 14 | K. W. A. E. | Systol. Murmur | 101.8 | 23,000 | — | | |
| B-902 | 25 | M | | | 5 | K. A. S. E. | Gallop rhythm | 102.2 | 16,000 | — | | Acute nephritis; p-r. interval 0.2; left hospital with pulse rate 110, temperature 99.6. |
| B-921 | 35 | F | 1 | | 6 | A. K. W. F. | Myo. Endo. & Pericard. | 104.6 | 10,400 | — | | |
| B-350 | 9 | M | 3 | | 6 | K. H. E. | Endocard. | 102.8 | 13,600 | — | | |
| B-918 | 44 | F | 1 | | 8 | H. K. A. W. | | 103.2 | 15,600 | — | | Furunculosis. |
| B-919 | 46 | F | 1 | Yes | 22 | S. A. | Endocard. Myocard. | 104.0 | 13,200 | — | | Acute otitis media. |
| B-348 | 11 | M | | | 42 | K. | Endocard. | 101.0 | 8,000 | — | | |
| B-40 | 35 | F | | | 10 | K. A. | | 105.4 | 15,500 | RB-5 | — | Schönlein's disease; many large patches of purpura on legs. |
| B-1423 | 20 | F | 1 | | 14 | W. A. K. F. | | 103.0 | | — | | |
| B-1412 | 29 | M | 1 | | 4 | A. K. W. | | 103.4 | | — | | |
| B-1433 | 29 | M | 1 | | 3 | K. S. F. A. | | 102.0 | | — | | |

*Cases Studied in 1929*

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| H. E. | 22 | F | 4 | | | F. K. W. | Endo. Peri. & Myocard. | 105.6 | 20,000 | RB-10 | — | No petechiae; beginning decompensation; spleen not enlarged; p-r. interval 0.24 right axis deviation; inverted T 1st & 2nd. |
| B-963 | 23 | M | | Yes | 5 | K. A. W. | Endocard. | 105.0 | 15,800 | RB-23 | | |

TABLE 1—*Concluded*

| Patient | Age | Sex | Previous Attacks | Preceding Sore Throat | No. of Days Ill Before Adm. | Joints* Involved | Cardiac Involvement § | Max. Tem. | W.B.C. | Blood Culture† | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | 1st | 2nd | |
| B-964 | 25 | F | | | 14 | K. A. S. | | 101.0 | | RB-22 | | |
| B-402 | 46 | M | | Yes | 5 | K. A. S. H. | | 101.4 | | RB-28 | | |
| B-384 | 29 | F | | Yes | 42 | F. E. K. S. | Endocard. | 101.8 | | RB-14 | | |
| B-861 | 38 | M | 1 | | 13 | W. K. A. | | 104.6 | 16,900 | RB-19 | | |
| B-965 | 48 | M | Many | | 11 | K. A. W. E. | Pulsus Bigem; Myocard. | 102.6 | | RB-20 | | |
| B-961 | 17 | M | Many | Yes | 30 | S. E. K. | Endo. & Pericard.; Auricul. Fibrill. | 104.0 | 18,600 | RB-24 | | Died suddenly; no autopsy. |
| B-1495 | 20 | M | 2 | Coryza | 14 | K. E. A. | Endocard. | 102.4 | | RB-35 | | Chorea as a child. |
| B-966 | 21 | F | | Yes | 21 | K. A. W. | Endocard. | 102.0 | | — | | |
| N. F. | 30 | F | 1 | Yes | 30 | K. E. A. | Endocard. | 102.4 | | RB-33 | | |
| P. S. | 35 | F | | Yes | 30 | K. S. W. F. E. | Endocard. | 104.0 | | RB-34 | — | S. V. (RJ-34) recovered from joint culture. |
| R. G. | 18 | M | 1 | Yes | 4 | A. K. V. | Peri. Endo. & Myocard. | 104.8 | | RB-32 | | Pleuritis with effusion p-r. interval 0.22. |
| D. S. | 44 | M | 1 | Yes | 5 | K. W. E. S. | Endo. & Pericard.; Auricul. Fibrill. | 103.0 | | RB-25 | | S. V. (RJ-25) recovered from joint culture. |
| B-1497 | 43 | F | | | 2 | S. E. W. F. K. | Systol. Murmur | 102.8 | | RB-21 | | |
| S. T. | 23 | M | | | 5 | W. E. H. K. A. | | 101.8 | | RB-11 | | |
| R. C. | 65 | M | 2 | | 7 | K. E. W. A. | Myocard. Endocard. | 103.2 | | — | | S. V. (RJ-36) recovered from joint culture. |
| B-400 | 38 | M | | Yes | 5 | K. S. E. A. | | 103.8 | | $RB\text{-}9_1$ | $RB\text{-}9_2$ | |
| A. K. | 29 | M | | Yes | 6 | K. A. | | 100.6 | | RB-17 | | S. V. (RJ-17) recovered from joint culture. |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M. B. | 46 | F | 1 | Coryza | 7 | K. S. E. A. | Endocard. | 103.4 | | RB-29 | | p-r. interval 0.15. |
| R. Z. | 32 | F | 1 | Yes | 60 | W. K. F. | Myocard. | 100.8 | 8,800 | RB-31 | — | Epilepsy. |
| N. M. | 14 | M | | | 5 | A. W. K. | Endocard. | 102.4 | 15,000 | RB-13 | | |
| S. F. | 9 | F | 1 | Yes | 3 | A. K. | Endocard. | 103.0 | 9,100 | RB-27 | | |
| L. M. | 47 | M | | | 3 | F. A. W. K. | Systol. Murmur | 104.0 | 6,900 | RB-16 | — | S. V. (RJ-16) recovered from joint culture. |
| A. F. | | M | 1 | Yes | 1 | S. W. K. | Endocard. Myocard. | 103.8 | | RB-18 | | |
| B-2304 | 28 | F | Many | Yes | 30 | W. K. | Endocard. | 103.4 | | RB-15 | | |
| A. C. | 13 | F | 1 | Yes | 7 | K. A. | Endocard. | 100.8 | | — | | |
| B-953 | 49 | M | Many | | 7 | A. K. S. H. | | 102.8 | 16,600 | RB-8 | | |
| B-1506 | 30 | M | 2 | | 30 | K. A. W. S. | | 101.8 | | RB-26 | | Wassermann ++++. Joint culture negative. |
| C. W. | 19 | M | 2 | | 2 | K. S. H. | Endocard. | 101.9 | | — | | Joint culture negative. |
| J. M. | 27 | M | | | 3 | K. S. E. F. | | 103.6 | | — | | |



* A, indicates ankle; E, elbow; F, fingers; H, hips; K, knee; S, shoulder; T, toes; W, wrist; V, vertebrae.

† Cultures positive for streptococci are indicated by the strain numbers. — = no growth.

§ Systol. murmur = Systolic murmur; Endocard. = Endocarditis; Pericard. = Pericarditis, Myocard. = Myocarditis; Auricul. Fibrill. = Auricular Fibrillation.

night. On the following morning, the clots are loosened with a glass rod and the tubes placed in the centrifuge. After centrifugalization, the serum is carefully removed with a pipette. The clots are broken up with glass tubes one-quarter inch in diameter, drawn up in the tubes and transferred to two bottles, each containing 50 c.c. of broth. The bottles are then covered with paper caps and placed in the incubator at 37 deg. C. for one month. During the month, the bottles are opened at five day intervals, and sub-cultures made in both blood-agar pour plates and blood broth tubes, using one-tenth c.c. of the original broth culture in each case. If no growth has been found at the end of a month, the original broth bottles are centrifugalized and the sediment cultured in fresh blood broth. Sub-cultures are made from the latter on blood agar plates several

TABLE 2

*Summary of Bacteriological Findings in Blood and Joint Cultures of Patients with Acute Rheumatic Fever (1928 and 1929 Series)*

| Organism found | Rheumatic fever | | Controls | |
|---|---|---|---|---|
| | Blood | Joints | Blood | Joints |
| Strep. viridans | 33 | 5 | 1 | 0 |
| Strep. hemolyticus | 1 | 0 | 0 | 0 |
| Strep. anhemolyticus | 1 | 0 | 0 | 0 |
| Total showing strep | 35 | 5 | 1 | 0 |
| Sterile | 25 | 2 | 65 | 13 |

days later, and if no organisms are found, the bottles are discarded and the culture considered negative. All transfers and manipulations are carried out under a hood.

(5) *Technic for Joint Cultures.*—Syringes and needles for aspiration are prepared in the same way as for blood cultures. The knee joint has been used for obtaining joint fluid. The skin over the joint is prepared with two coats of tincture of iodin and one of alcohol. Two per cent novocain is injected down to the capsule to render the parts anesthetic. A 20-gauge needle and a 20 c.c. syringe are usually employed for the aspiration. The fluid from the joints is cultured in three or four blood broth tubes, using varying amounts for each tube. The tubes are placed in the incubator for one month. Sub-cultures are made every few days in blood-agar pour plates and in blood-agar streak plates. Streptococci usually make their appearance during the first week, but the cultures are not pronounced sterile until they have been in the incubator one month.

### *Results of Blood Cultures*

Protocols of the 60 patients on whom blood cultures and, in some instances, joint cultures were made, are listed in Table 1. The age of

these patients varied from 7 to 65 years. Thirty-four gave a history of one or more previous attacks of rheumatic fever. In 26 patients a history was given of a preceding sore throat or coryza. Every patient in the series gave a history of joint manifestations, and in nearly every case several joints were still obviously inflamed at the time the cultures were taken. Every case showed fever varying from a maximum of 100.4 to 105.6. Leucocytosis was present in most of the cases on whom counts were made. Only three patients in the

TABLE 3

*Patients with Rheumatic Fever Subjected to Two Blood Cultures*

| Patient | 1st Blood Culture | 2nd Blood Culture | No. of Days Between Cultures | Status of Disease at Time of 2nd Blood Culture |
|---|---|---|---|---|
| B-356 | + | − | 5 | Active |
| B-924 | + | − | 10 | Inactive |
| B-40 | + | − | 2 | Subsiding |
| B-948 | − | + (P.M.) | 2 | Patient dead |
| H. E. | + | − | 57 | Inactive |
| P. S. | + | − | 21 | Subsiding |
| R. Z. | + | − | 50 | Inactive |
| L. M. | + | − | 21 | Subsiding |
| B-400 | + | + | 9 | Active |

Active indicates that the patient had high fever and joint lesions.

Subsiding indicates that the fever was subsiding and the patient recovering from the attack.

Inactive indicates that the patient had no fever and was out of bed.

series of 60 cases died. One came to autopsy and showed no evidence of bacterial endocarditis. In the other two fatal cases, the opinion of all those who studied them clinically was that death was due to a serofibrinous pericarditis and not to a bacterial endocarditis.

Of the 29 cases studied by blood culture in 1928, nine, or 31 per cent, were found, after varying periods of incubation, to contain a gram-positive micrococcus in pure culture. Of the 31 cases studied in 1929, 26 or 83.9 per cent yielded a micrococcus in pure culture. The organisms appeared in pairs or short chains and had all the morphologic characteristics of streptococci (Table 2). The higher

percentage of positive cultures obtained in 1929 is probably traceable in large part to improvements in technic, though a personal equation was also present, the cultures in 1928 having been examined by one of the writers and the cultures in 1929 by another.

Nine patients studied in this series were subjected to two blood cultures (Table 3). Of these patients 8 showed streptococci in the

TABLE 4

*Classification of Patients Studied by Blood Cultures*

| | No. of cases Studied by Blood cultures | Total | Streptococcus in Blood cultures |
|---|---|---|---|
| Acute Rheumatic Fever | 60 | 60 | 35 |
| Controls: | | | |
| Fever of unknown origin (Blood cult. positive) | 1 | | |
| Degenerative arthritis | 23 | | |
| Gonococcal arthritis | 4 | | |
| Chronic myositis | 7 | | |
| Chronic neuritis | 3 | | |
| Gout | 2 | | |
| Chorea | 2 | | |
| Tuberculous adenitis | 2 | | |
| Diabetic neuritis | 1 | 66 | 1 |
| Convalescent pneumonia | 7 | | |
| Iridocyclitis | 1 | | |
| Weak foot | 1 | | |
| Chronic colitis | 1 | | |
| Sciatica | 1 | | |
| Gastric neurosis | 1 | | |
| Chronic nephritis | 1 | | |
| Neurasthenia | 3 | | |
| Normal individuals | 5 | | |

first culture; two yielded streptococci in the second culture. All blood cultures taken when the disease was subsiding or inactive were sterile.

*Other Bacteria Isolated from Blood Cultures.*—Some type of streptococcus was the only organism consistently recovered from blood cultures. In one instance blood cultures yielded a diphtheroid bacillus, but in view of the frequency in which this organism is found as a

contaminant, no significance was attached to its presence. All grossly contaminated cultures were discarded.

*Control Blood Cultures.*—It seemed important to check these observations on the blood of patients with acute rheumatic fever by a series of blood cultures on patients suffering from other diseases and on normal individuals. Altogether, 66 controls were studied by means of blood cultures, the technic being similar in all respects to that used in acute rheumatic fever (Table 4).

All the controls, with one exception, yielded sterile blood cultures.

The exceptional case (B-284) was that of a man, aged 53, who six hours before admission began to have muscular pains particularly in the back and legs associated with malaise and high fever. There were no other symptoms. The past history was negative. Physical examination showed a flushed feverish man with a reddened pharynx. The remainder of the examination was negative. X-rays of the sinuses, lungs and gall-bladder revealed no evidence of disease. W.B.C. 6200; Widal negative; urine negative. The temperature on admission was 104.6. It came down to normal on the fifth day. Then it gradually rose again, and ran an irregular course between 100 and 102 for another four weeks, when it came down to normal and remained there. The final diagnosis on this patient was "Fever of unknown origin." A blood culture taken on the day of admission yielded a pure culture of streptococcus viridans.

TABLE 5

*Classification of Patients Studied by Joint Cultures*

| | No. of cases Studied by Blood culture | Total | Streptococcus in Joint cultures |
|---|---|---|---|
| Acute Rheumatic Fever | 7 | 7 | 5 |
| Controls: | | | |
| Degenerative arthritis | 4 | 13 | 0 |
| Gout | 1 | | |
| Chronic synovitis | 3 | | |
| Traumatic synovitis | 4 | | |
| Osteitis fibrosa cystica | 1 | | |

## *Results of Joint Cultures*

In seven cases of acute rheumatic fever, the opportunity presented itself for culturing the synovial fluid from one of the affected joints.

In every case, the knee was the joint selected for puncture, and in every instance 5 to 20 c.c. of slightly cloudy, straw-colored fluid was removed. Of the seven cases, five yielded a streptococcus in pure culture after varying periods of incubation (Table 5). Neither of the two patients on whom sterile joint cultures were obtained were presenting very active symptoms at the time the punctures were made.

*Control Cultures.*—These results were checked with joint cultures of patients suffering from other diseases (Table 5). Altogether, 13 controls were studied by means of joint cultures, the technic being similar in all respects to that used on patients with acute rheumatic fever. All the controls yielded sterile cultures.

### *Cultural and Biologic Characteristics of the Streptococci Isolated from Blood and Joint Cultures*

The average time of appearance of streptococci in the blood cultures was approximately 17 days; in joint cultures, 8 days. In one instance the blood culture had been in the incubator six weeks before streptococci could be demonstrated. On the other hand there were exceptional cases in which the streptococci appeared in three or four days. In many instances, the first colonies observed on the blood agar sub-culture plates failed to grow when seeded into blood broth tubes. It was often necessary to return to the original broth cultures several times before successful transfer of colonies to blood broth could be achieved.

The streptococci first make their appearance in broth as pairs or very short chains, the chains becoming longer as the organisms became more accustomed to laboratory mediums. Generally, the organisms grow very poorly for the first few generations, necessitating frequent transfers of cultures. In blood agar sub-cultures, both superficial and deep colonies are usually recognizable after 24 to 48 hours of incubation.

The streptococci encountered in the blood and joint cultures with two exceptions belong to the alpha streptococcus or streptococcus viridans group. One exception proved to be a typical hemolytic or beta streptococcus, while the other was classified as a non-hemolytic or gamma streptococcus. All the streptococci isolated were gram-positive and insoluble in bile.

Morphologically, the streptococcus viridans strains usually appear as very small, round or slightly oval cocci, which in liquid mediums form pairs or very short chains. On blood agar plates, after 48 hours incubation, deep colonies appear as minute, biconvex grayish colonies surrounded by a very small zone of incomplete hemolysis that contains specks of green pigment. The amount of methemoglobin formation varies considerably with different strains. A few strains show a large amount of green pigment, but as a general rule, the amount of green produced is small.

No hemolysis of the blood occurs in blood broth cultures after 48 hours incubation. The broth becomes turbid and takes on a slightly greenish tinge.

The strain of streptococcus hemolyticus cultivated from one patient grows readily on all the usual laboratory mediums. In blood agar, after 48 hours incubation, deep colonies appear as moderate-sized, biconvex grayish colonies, surrounded by large zones of complete hemolysis. The indifferent streptococcus isolated from one patient appears as a small, round or slightly oval coccus, which in liquid mediums form very short chains. On blood agar, after 48 hours of incubation, deep colonies appear as small, biconvex grayish colonies with no change in the surrounding medium. There is no hemolysis of the blood in blood broth cultures.

*Sugar Fermentations.*—Sugar fermentations were carried out on 32 strains of streptococci isolated from the blood, and five strains isolated from the joints (Table 6).

The tests were made in 5 c.c. of sugar free broth, to which 0.1 c.c. of a ten per cent solution of one of the sugars had been added. The pH. of the sugar mediums was uniformly 7.6. Each tube was heavily inoculated with an actively growing 24-hour broth culture. The tubes were allowed to incubate for five days, and the amount of acid formation determined by the pH. method. Tubes that gave only a small amount of fermentation were allowed to incubate for an additional 48 hours, but no further amount of acid formation was noted in any case.

Thirty-four of the 37 strains fermented lactose, mannit and salicin. According to Holman's classification, this group would be classified as streptococcus fecalis It included all but one of the streptococcus viridans strains. Three of the 37 strains (RB-2, RB-5 and RB-6) fermented salicin and lactose, but had no effect on mannit. Of these three strains, two were obviously members of a different biological group. Strain RB-2 was a streptococcus hemolyticus and RB-5 was

a gamma or indifferent streptococcus. Only two strains in the entire series produced acid in inulin, and even in these two the change in the pH. was so slight (7.2) that the strains could hardly be called inulin fermenters.

It is significant that three strains of streptococcus viridans isolated from joints corresponded in their fermentation reactions with the strains isolated from the blood in the same patients. Strains RB-$9_1$ and RB-$9_2$, isolated at different times from the blood of Case B-400, gave identical sugar reactions.

TABLE 6

*Sugar Reactions of Streptococci*

| Strain | Dextrose | Salicin | Lactose | Mannit | Inulin | Strain | Dextrose | Salicin | Lactose | Mannit | Inulin |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RB-1 | 6.2 | 6.8 | 6.2 | 6.4 | 7.6 | RB-22 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-3 | 6.2 | 6.8 | 6.0 | 6.0 | 7.6 | RB-23 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-7 | 6.5 | 6.8 | 6.0 | 6.2 | 7.6 | RB-24 | 6.2 | 6.8 | 6.0 | 6.2 | 7.6 |
| RB-8 | 6.0 | 6.0 | 6.0 | 7.0 | 7.6 | RB-25 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-$9_1$ | 6.0 | 6.4 | 6.0 | 6.0 | 7.6 | RJ-25 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-$9_2$ | 6.2 | 6.8 | 6.0 | 6.2 | 7.6 | RB-27 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-10 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-28 | 6.0 | 6.4 | 6.0 | 6.0 | 7.6 |
| RB-11 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-29 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-12 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-31 | 6.0 | 7.0 | 6.0 | 6.0 | 7.6 |
| RB-13 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-32 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-14 | 6.0 | 6.4 | 6.4 | 6.2 | 7.6 | RB-34 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 |
| RB-15 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RJ-34 | 6.0 | 6.0 | 7.0 | 6.0 | 7.6 |
| RB-16 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RJ-36 | 6.0 | 6.6 | 6.0 | 6.0 | 7.6 |
| RJ-16 | 6.0 | 7.0 | 6.0 | 7.0 | 7.6 | RB-26 | 6.0 | 6.0 | 7.0 | 6.0 | 7.2 |
| RJ-17 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-35 | 6.0 | 6.0 | 6.0 | 6.0 | 7.2 |
| RB-18 | 6.0 | 6.6 | 6.0 | 6.0 | 7.6 | RB-2 H | 6.0 | 6.0 | 6.0 | 7.6 | 7.6 |
| RB-19 | 6.2 | 6.4 | 6.0 | 6.2 | 7.6 | RB-5 nH. | 6.0 | 6.8 | 6.2 | 7.5 | 7.6 |
| RB-20 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | RB-6 | 6.0 | 6.0 | 7.0 | 7.6 | 7.6 |
| RB-21 | 6.0 | 6.0 | 6.0 | 6.0 | 7.6 | | | | | | |

The figures in the table indicate pH reading after 5 days incubation.
Readings of 7.6 = no acid formation.
Readings of 7.0 or below = definite acid formation.

*Agglutination Reactions.*—In order to determine whether there was any biologic relationship between the streptococci which had been isolated from the blood and joints of patients with acute rheumatic fever, cross agglutination tests were made with 16 serums from

rabbits immunized against 16 strains of streptococci. Fifteen of these strains were from the blood or joints of patients with rheumatic fever. The sixteenth strain was a streptococcus (AB-4) isolated from the blood of a case of chronic infectious arthritis.

The 16 immune serums were tested for agglutinins against each of the 35 strains of streptococcus recovered from patients with rheumatic fever and against one strain from a case of chronic infectious arthritis. In addition the serums were tested against a streptococcus viridans recovered from the blood of a patient with subacute bacterial endocarditis.

The agglutination tests were carried out as follows:

A 24-hour broth culture was killed by heating at 56 C. for two hours, and the centrifugalized sediment re-suspended in physiological salt solution. The rabbits received 5 c.c. daily for five days. After five days rest, they were given five further injections (10 to 15 c.c. each) at daily intervals. After another five days rest, tests for the agglutination titer were made.

By this method, the serums when collected were usually found to be rich in agglutinins. However, in a few instances, it was necessary to give additional injections of streptococci. All the serums showed a minimum agglutination titer of 1:2560.

The antigens for the agglutinations were prepared by growing the streptococci in plain broth for eighteen hours. A diffuse growth was obtained in this way with every strain. 0.5 c.c. of the antigen was mixed with 0.5 c.c. of the various dilutions of immune rabbit's serum. The agglutinations were carried out to a titer of at least 1:2560, and sometimes to a titer of 1:655,000. As controls, rabbit's serum withdrawn before immunization was tested against the streptococci. Another control consisted of 0.5 c.c. of sterile broth and 0.5 c.c. of the bacterial antigen. The agglutination tubes were left two hours in the water bath at 56 C. They were then placed in the refrigerator, and readings were made the following morning.

Examination of Table 7 reveals the fact that these green streptococci recovered from the blood and joints of rheumatic fever patients show a definite tendency to fall into biological groups. We have indicated this grouping in the chart by rectangles. These streptococcus groups differ from pneumococcus groups in that there is more tendency to partial cross-agglutination between the groups. This is particularly noticeable in the case of the dominant group in the upper left corner of the chart. There are several strains listed just below this group

TABLE 7

*Agglutination Tests*

*Agglutination Reactions with Serums of Rabbits Immunized against Fifteen Streptococcus Strains from Rheumatic Fever and One from Chronic Infectious Arthritis*

| Strains | Serum RB-19 | Serum RB-24 | Serum RB-9 | Serum RB-7 | Serum RB-25 | Serum RJ-36 | Serum RB-5 | Serum RB-16 | Serum RB-22 | Serum RJ-17 | Serum RJ-34 | Serum RB-26 | Serum RB-4 | Serum RB-1 | Serum RB-2 | Serum AB-4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RB-19 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-24 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-9 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-9(2) | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-14 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-18 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-13 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-7 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-25 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RJ-25 | 4 | 4 | 4 | 4 | 4 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-20 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| RB-36 | 3 | 3 | 3 | 2 | 4 | 4 | 2 | 3 | 2 | 4 | 0 | 1 | 2 | 2 | 0 | 0 |
| RB-3 | 2 | 2 | 3 | 2 | 2 | 3 | 2 | 3 | 4 | 3 | 1 | 0 | 1 | 1 | 0 | 0 |
| RB-8 | 2 | 2 | 2 | 2 | 3 | 2 | 2 | 3 | 2 | 4 | 4 | 1 | 2 | 1 | 0 | 0 |
| RB-5 | 2 | 2 | 2 | 2 | 3 | 4 | 4 | 3 | 2 | 3 | 1 | 4 | 1 | 1 | 0 | 0 |
| RB-30 | 1 | 1 | 2 | 1 | 0 | 0 | 1 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-31 | 2 | 2 | 2 | 2 | 0 | 0 | 2 | 3 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| RB-4 | 2 | 1 | 2 | 2 | 0 | 0 | 3 | 0 | 2 | 0 | 0 | 0 | 4 | 2 | 0 | 0 |
| RB-15 | 2 | 2 | 2 | 2 | 0 | 0 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-16 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 1 | 1 | 0 | 2 | 1 | 0 | 0 | 0 |
| RJ-16 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 3 | 1 | 2 | 0 | 1 | 1 | 0 | 0 | 0 |
| RB-32 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-29 | 1 | 1 | 0 | 0 | 0 | 2 | 1 | 4 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 |
| RB-28 | 1 | 1 | 0 | 0 | 0 | 0 | 2 | 3 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| RB-34 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 3 | 0 | 0 | 1 | 0 | 0 |
| RJ-34 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 4 | 0 | 0 | 0 | 0 | 0 |
| RB-35 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| RJ-17 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-26 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 |
| RB-2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 2 | 0 | 4 | 4 |
| RB-22 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 1 | 0 | 2 | 0 | 2 | 1 |
| RB-23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-10 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-27 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| RB-1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 3 | 4 | 0 | 0 |
| S. B. E. | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 3 | 0 | 0 | 4 | 4 | 0 | 0 |
| AB-4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 4 | 4 |

For the agglutination tests the following dilutions of serums were used, 1:20 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560. 4 = Complete agglutination with sedimentation; 3 = agglutination and sedimentation with slight turbidity; 2 = agglutination without sedimentation, 1 = slight agglutination, 0 = no agglutination.

RB = Rheumatic fever strains; AB = Chronic infectious arthritis strains; S. B. E. = Subacute bacterial endocarditis. The larger groups are indicated with rectangles.

that appear to be closely related to it, though they fail to show complete agglutination reactions. The immune serum of hemolytic streptococcus, RB-2, produced little or no agglutination of any of the other rheumatic fever strains, but strongly agglutinated the strain AB-4 from a case of chronic infectious arthritis. Likewise, there was no agglutination between the immune serum of strain AB-4 and the rheumatic fever strains, except the hemolytic streptococcus RB-2. These two strains appeared to be biologically identical. Two of the immune serums gave a complete cross-agglutination and three a partial agglutination with the strain of streptococcus viridans recovered from a case of subacute bacterial endocarditis.

One of the most interesting features brought out in the agglutination studies was the close biologic relationship between the streptococci isolated from the blood and from the joints of the same patient. From three of the patients in this series, streptococci have been recovered from both the blood and the joints, and in every instance the strain recovered from the joint showed a high degree of cross-agglutination with the strain recovered from the blood.

It is also significant that in the case of patient B-400, two blood cultures, taken nine days apart, yielded streptococci that cross-agglutinated.

*Absorption Tests.*—Absorption tests were carried out on each immune rabbit serum with the strains that showed complete agglutination, and in several instances where there was only partial agglutination. The method was as follows:

> The sediment from 300 c.c. of a 24-hour broth culture of a streptococcus was mixed with 0.5 c.c. of a 1:10 dilution of the immune serum. The mixture was shaken and placed in the water bath for two hours at 56 deg. C. While in the water bath, it was agitated at frequent intervals. It was then placed in the ice-box overnight. In the morning, the mixture was centrifugalized and the supernatant fluid removed. Agglutination tests were set up, using the same dilutions as in the original agglutination tests. Controls were carried out with unabsorbed serum and normal rabbit's serum. All the tubes were placed in the water bath at 56 deg. C. for two hours. After standing in the ice-box overnight readings were made.

Complete absorption occurred in practically every case where there had been complete agglutination (Table 8). In the group containing

TABLE 8

*Absorption Tests*

*Amount of Agglutination of Streptococcal Strains with Serums of 11 Immunized Rabbits before and after Absorption of Agglutinins*

| Strain | RB-19 | | RB-24 | | RB-9 | | RB-7 | | RB-25 | | RJ-36 | | RB-22 | | RB-16 | | RJ-34 | | RJ-17 | | RB-26 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. | Un. | A. |
| RB-19 | 4 | 0 | 4 | 0 | 4 | 0 | 4 | 0 | 4 | 0 | | | | | | | | | | | | |
| RB-24 | 4 | 0 | 4 | 0 | | | | | | | 4 | 0 | | | | | | | | | | |
| RB-9 | 4 | 0 | | | 4 | 0 | | | | | 4 | 0 | | | | | | | | | | |
| RB-9(2) | 4 | 0 | | | | | | | | | | | | | | | | | | | | |
| RB-14 | 4 | 0 | | | | | | | | | | | | | | | | | | | | |
| RB-18 | 4 | 0 | | | | | | | | | | | | | | | | | | | | |
| RB-13 | 4 | 0 | | | | | | | | | | | | | | | | | | | | |
| RB-7 | 4 | 0 | | | | | 4 | 0 | | | | | | | | | | | | | | |
| RB-25 | 4 | 0 | | | | | | | 4 | 0 | 4 | 0 | | | | | | | | | | |
| RJ-25 | 4 | 0 | | | | | | | 4 | 0 | | | | | | | | | | | | |
| RB-20 | 3 | 1 | | | | | | | | | | | 4 | 0 | | | | | | | | |
| RB-31 | 2 | 2 | | | | | | | | | | | | | 3 | 3 | 4 | 0 | | | | |
| RB-5 | 2 | 2 | | | | | | | | | 4 | 1 | | | 4 | 0 | | | | | 4 | 0 |
| RB-21 | 0 | 4 | | | | | | | | | | | | | 4 | 0 | | | | | | |
| RJ-36 | | | | | | | | | | | 4 | 0 | | | | | | | 4 | 2 | | |
| RB-32 | | | | | | | | | | | | | | | 4 | 0 | | | | | | |
| RJ-16 | | | | | | | | | | | | | | | 4 | 0 | | | | | | |
| RB-15 | | | | | | | | | | | | | | | 4 | 0 | | | | | | |
| RB-29 | | | | | | | | | | | | | | | 4 | 0 | | | | | | |
| RB-27 | | | | | | | | | | | | | 4 | 0 | | | | | | | | |
| RB-2 | | | | | | | | | | | | | 4 | 2 | | | | | | | | |
| RB-10 | | | | | | | | | | | | | 4 | 0 | | | | | | | | |
| RB-23 | | | | | | | | | | | | | 4 | 0 | | | | | | | | |
| RB-22 | | | | | | | | | | | | | 4 | 0 | | | | | | | | |
| RJ-34 | | | | | | | | | | | | | | | | | 4 | 0 | | | | |
| RB-34 | | | | | | | | | | | | | | | | | 3 | 0 | | | | |
| RB-8 | | | | | | | | | | | | | | | | | 4 | 0 | 4 | 2 | | |
| RB-28 | | | | | | | | | | | | | | | | | 4 | 1 | | | | |
| RB-26 | | | | | | | | | | | | | | | | | | | | | 4 | 0 |
| RB-17 | | | | | | | | | | | | | | | | | | | 4 | 0 | | |
| S. B. E. | | | | | | | | | | | | | | | | | | | 3 | 1 | | |

In the absorption tests, the following dilutions of serums were used for the agglutination reactions; 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560. 4 = complete agglutination with sedimentation; 3 = agglutination and sedimentation with slight turbidity of the broth: 2 = agglutination without sediment; 1 = slight agglutination; 0 = no agglutination.

Un. = Unabsorbed serum. A. = Absorbed serum.

ten homogeneous strains, the agglutinins in the homologous immune serums were completely absorbed in every instance. This establishes the identity of these strains. The smaller groups indicated in the agglutination table can also be identified in the absorption table. (See absorption reactions with immune serums, RB-22 and RB-16.)

*Relation of Streptococci Recovered from the Blood to Streptococci Isolated from the Joints*

In 7 rheumatic fever patients who were subjected to joint cultures, 5 yielded a streptococcus, in every instance of the alpha or viridans type. In 4 of these 5 patients, streptococci culturally identical with those recovered from the joints were recovered from the blood cultures. Unfortunately one of the bloodstream streptococci was lost in subcultures. In the remaining three cases in which streptococci were isolated from both the blood and the joint, agglutination and absorption tests have showed them to be biologically identical (Tables 7 and 8). It appears from these findings that the joints in rheumatic fever are actually infected, and that the streptococcus responsible for the infection is the same strain that circulates in the blood.

## DISCUSSION

In reviewing the literature dealing with the bacteriology of rheumatic fever, it has been pointed out that the results obtained by different investigators who have cultured the blood and the joints in this disease have varied from entirely negative results to a high percentage of positive results. It is significant, however, that when the results have been positive, the organism isolated has almost invariably been some form of streptococcus. That the problem is one almost entirely of technic is evident from a comparison of our own results in the Spring of 1928 with our results in the Spring of 1929. In 1928 the percentage of blood cultures positive for streptococci was 31 per cent. In the 1929 series, the percentage of positive streptococcus blood cultures was 83.9 per cent. If one goes still further and asks: What are the essential factors in the successful cultivation of streptococci from the blood and joints in rheumatic fever, the answer is not easy. Most important of all are technical

competence and experience, obtained preferably by work with streptococci. The first generation of these micro-organisms on artificial media is cultivated with great difficulty. Something is present in the medium which retards growth to a marked degree. In blood agar plates deep colonies are easily overlooked with the naked eye, and much help will be derived from a magnifying lens. As for the culture medium itself, an accurate titration of the pH. to 7.6 is important. Just how much of a factor is the particular beef-heart, tap water or peptone employed we are not prepared to say.

The organisms which have been isolated from the blood and joints in this series of cases appear to differ in no respect from those recovered by previous investigators. All but two strains have been classified as alpha streptococci (Str. viridans); one strain was classified as a beta streptococcus (Str. hemolyticus); one strain had no effect whatever on blood agar and was classified as a gamma streptococcus (Str. anhemolyticus). The amount of methemoglobin produced by the alpha streptococci varied considerably with the strain, some strains producing only a small amount. Even in these latter strains, however, a slight degree of hemolysis was produced on blood agar plates after 48 hours of growth, a characteristic which prevented our confusing them with gamma streptococci.

In this connection, a word should be added as to the bearing of the results herein reported on the work of Small (18) and of Birkhaug (21). Both of these investigators had their interest in the indifferent or gamma streptococci first aroused by the recovery of such an organism from the bloodstream of a patient with rheumatic fever. Small called his organism the "streptococcus cardioarthritidis;" Birkhaug referred to his strain as a "non-methemoglobin-forming" streptococcus. The evidence presented by these investigators in favor of the indifferent streptococcus as the exciting agent in rheumatic fever was based not so much on the successful recovery of this organism from the blood, joints and other lesions of rheumatic fever as it was on the frequent cultivation of gamma streptococci from the tonsils, throat and feces of patients with the disease. Hitchcock (22), however, has recently shown that indifferent streptococci are as frequent inhabitants of the throats of nonrheumatics as of rheumatic individuals. In our experience, as well as in that of other investigators who

have cultivated streptococci from the lesions of rheumatic fever, the streptococcus viridans plays a much more important rôle than the indifferent streptococcus. It is true that a number of the strains which we have classified as alpha streptococci might have been placed in the gamma group by other investigators. The criteria which we have employed compelled us to assign these organisms to the alpha group. The possibility, of course, of mutations from one form of streptococcus into the other must not be overlooked.

It appears from this and previous studies, then, that various types of streptococci can act as excitants in rheumatic fever. Even the hemolytic streptococcus occasionally induces the symptoms and lesions of the disease. (See case B-375 in this series and one case in Clawson's series.) 38 strains of streptococcus viridans recovered from our series of patients with rheumatic fever have been found by agglutination and absorption tests to fall into a number of biological groups. The incidence and significance of these various groups will have to be determined by further study.

In our previously published studies on the bacteriology of the blood and joints in chronic infectious arthritis (20), it was shown by appropriate cultural and agglutination tests that the strains of streptococci isolated from affected joints were in each case culturally and biologically identical with the strain isolated from the patient's blood. The same holds true with respect to the streptococci obtained from the blood and joints in rheumatic fever. The serum of rabbits immunized against a streptococcus of blood culture origin has in every instance cross-agglutinated the streptococcus obtained by joint puncture in the same patient, and the degree of agglutination has been practically the same for the two strains.

The demonstration of streptococci in the blood and joints of a high percentage of patients with acute rheumatic fever makes it possible to draw certain analogies between this disease and other forms of arthritis, particularly gonococcal arthritis and infectious arthritis. In both these latter infections, a primary focus acts as a nidus for pathogenic bacteria which under certain circumstances break into the bloodstream and become localized in the joints where they set up metastatic infections. It has long been known that such a sequence of events takes place in gonococcal arthritis. In a recent study on

the rôle of the streptococcus in chronic infectious arthritis (20), we have presented evidence which goes far to show that a similar mechanism is at work in chronic infectious arthritis. From the findings reported in the present investigation it appears that rheumatic fever, at least in its acute stage, is a bacteremia and that streptococci circulate in the bloodstream and establish multiple secondary foci of infection in and about the joints.

During the last few years the rôle of allergy in the pathogenesis of rheumatic fever has been studied by Swift, Birkhaug, Zinsser and others, and much that is new and interesting has been added to our knowledge of this disease. Undoubtedly allergy plays an important part in rheumatic fever, just as it does in tuberculosis, syphilis and other infections. It is highly probable that patients infected with the rheumatic fever organism do not develop joint manifestations until a certain degree of susceptibility to this organism has been attained. Swift (23) points out, for example, that patients with rheumatic fever show a marked tissue reaction to streptococcus antigen, whereas patients with subacute bacterial endocarditis do not show the same state of reactivity to streptococci. During the hypersensitive state joint symptoms develop. On the other hand, in bacterial endocarditis, tissue immunity has been acquired and acute joint manifestations are rarely seen. Important, however, as these allergic phenomena are to a proper understanding of rheumatic fever, the fact must not be lost sight of that the demonstration of streptococci in the blood and in the joints of rheumatic fever patients provides convincing evidence that acute rheumatic fever is a bacteremia and that the joint manifestations are true infections differing in no essential respect from the metastatic joint manifestations observed in other bacteremias. Allergy undoubtedly exercises an important influence on the clinical manifestations of this infection. In both rheumatic fever and in bacterial endocarditis green streptococci are circulating in the bloodstream. In the former disease many joints become infected. In the latter disease, though the bloodstream usually contains more streptococci than are encountered in rheumatic fever, the joints remain free from infection. It is reasonable to suppose, therefore, that in rheumatic fever the patient's tissue is allergic to streptococci, while in infectious endocarditis the tissues

are immune to these organisms. A state of allergy toward the streptococcus, however, will probably not in itself induce the lesions or joint manifestations of rheumatic fever without the concomitant presence of streptococci. It is significant that vascular lesions and joint manifestations closely resembling those of rheumatic fever in man have been produced experimentally by several investigators by injecting streptococci of rheumatic origin into the ear veins of rabbits. So far, however, no one has produced the lesions or the joint manifestations of rheumatic fever in animals by the injection of streptococcus allergens.

## SUMMARY AND CONCLUSIONS

1. During the Spring of 1928, 29 patients with acute rheumatic fever were subjected to blood cultures, of whom 9, or 31 per cent, yielded a streptococcus. During the Spring of 1929, 31 patients with acute rheumatic fever were studied by blood cultures, of whom 26, or 83.9 per cent, yielded a streptococcus. The higher percentage of positive cultures in the 1929 series appears to have been due to improved cultural methods.

2. Of the 35 strains of streptococci recovered from blood cultures, 33 have been classified as alpha streptococci (Str. viridans); one as a beta streptococcus (Str. hemolyticus); and one a gamma streptococcus (Str. anhemolyticus). Some of the viridans strains produced very little green on blood media.

3. Agglutination and absorption tests indicate that the strains of streptococcus viridans recovered from the blood of patients with rheumatic fever show a tendency to fall into specific biological groups.

4. In 7 patients with rheumatic fever who were subjected to cultures from affected joints, 5, or 71.4 per cent, yielded a streptococcus viridans. In 3 patients in whom green streptococci were recovered from both the blood and joint, agglutination and absorption tests proved the identity of the strains isolated from the two sources.

5. These findings corroborate those of previous investigators and make it difficult to escape the conclusion that rheumatic fever is a streptococcal infection usually of the *alpha* or *viridans* type.

6. The pathogenesis of rheumatic fever in respect to the joint lesions appears to be analogous to that of infectious arthritis and

gonococcal arthritis. Bacterial allergy probably influences the clinical picture in all three conditions, but in each instance the joint manifestations are primarily dependent upon localization of bacteria in the joint, with subsequent infection.

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# NASOPHARYNGEAL FLORA IN HEALTH AND DURING RESPIRATORY DISEASE IN ISOLATED COMMUNITIES IN ALABAMA AND LABRADOR

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A report on the bacteriology of the nasopharynx in two isolated communities is given in this paper. The first community studied was in southern Alabama near the Gulf of Mexico, the second in the interior of Labrador. What was regarded as the normal flora was determined in each place. During the course of the studies, epidemics of nasopharyngitis or colds occurred in Alabama and of tracheitis in Labrador. These outbreaks were also studied bacteriologically.

The report embodied in this paper belongs naturally to the series of reports already published by Jordan, Park, Bloomfield, Noble and others on the nasopharyngeal flora in health and disease. The earlier studies were carried out in large population centers in which the environmental factors are numerous and uncontrollable.

In an effort to minimize the extraneous factors, we undertook the study of isolated communities, where life is simple, outside contacts infrequent, and environmental factors more accurately determinable and controllable.

## *Methods*

The method pursued was that of taking nasopharyngeal swabs by means of West tubes. The swabs were drawn from the glass tube with sterile forceps and placed in tubes containing 5 per cent blood hormone broth. When cultures were taken at a distance from the laboratory they were placed immediately in a water-jacketed container at 37°C. for transport. Usually the tubes were incubated at 37°C. for three hours in order to secure uniform incubation periods independent of transportation times and to suppress the Gram-negative cocci as well as to promote growth of pneumococci and Pfeiffer bacilli if present in small numbers.

After the preliminary incubation, the swabs were whipped vigorously in the medium and discarded. A loopful of blood broth was streaked on 3 to 5 per cent blood agar plates which were incubated for 36 to 48 hours. Representative type colonies were fished from the plates and the percentage of each type of colony present was estimated. The identification procedures were those in common use.

The liquid medium employed consisted of hormone broth (Fisk and Burky(1)). In Alabama beef infusion and in Labrador sheep hormone broth agar were also used as basic media. Hormone broth enriched with 1 per cent sheep serum and 1 per cent sugar with brom cresol purple as indicator served for satisfactory fermentation tests. In Labrador the pneumococci grew slightly or not at all in sheep hormone broth, but the addition of 1 drop of sterile sheep serum to 5 cc. of the broth provided the necessary accessory substance to produce luxuriant growth.

Rabbit, sheep and horse blood agar were employed at various times, the most satisfactory being rabbit blood agar medium. All organisms commonly found in the nasopharynx, particularly the Pfeiffer bacilli and the pneumococci, grow well on it, The pneumococcus colony is more characteristic than on the other kinds of blood media. Horse blood is also satisfactory but Pfeiffer bacilli grow less well and pneumococci form less characteristic colonies. Sheep blood is not satisfactory because the Pfeiffer bacilli grow poorly on it.

## *Places and Groups Studied*

### *Alabama*

The first study was conducted in southern Alabama from November 1927 to May 1928. The community selected consisted of about twenty-five families living in small clearings in the forest along Patsiliga River and named appropriately Happy Hollow. The cultures were taken from the school children. This district is well isolated from the trading centers of Andalusia and Greensville both by distance (20 miles) and the poor condition of the roads. The visits of the male adults to the more populous centers are confined to occasional trading visits and semiannual attendance at court, when the women sometimes accompany them. The children rarely go to town. The school teacher makes monthly visits to town to attend a teachers' conference. Few outsiders visit the region. The economic status of the people is poor.

The Happy Hollow school is a well-ventilated one room frame building, heated when necessary by a wood-burning stove. It seats without crowding its twenty-three pupils whose ages range from six

to sixteen. All children and the teacher live within three miles of the school. The teacher, a capable woman, supplied much of the requested information.

Seven sets of cultures were taken at this school at irregular intervals. They have been divided on the basis of clinical condition into the following groups:

(*1*) *Period of Normal Health.* In November and December two sets of cultures were taken when all members of the group were in normal health.

(*2*) *Period of Acute Colds.* In January there was a marked fall in temperature to below freezing. This period of cold (unusual for this subtropical region) continued for about two weeks, after which there was a considerable rise in temperature. At about this time the teacher returned from a week-end visit to Andalusia, where, according to her statements, she acquired a cold which was so severe that she likened it to influenza, which she had had during the epidemic period of 1918. The first cultures in this period were obtained about three days after her return. At this time four children also had colds that had developed during the preceding few days. One week later every person in the school had a cold, and another set of cultures was taken. One week later another set was taken. At this time a few individuals had recovered, but the great majority had coughs with varying amounts of nasal discharge.

(*3*) *Period of Recovery.* One set of cultures was obtained during March, one month after the cold epidemic had reached its height. At this time every one seemed normal. In April another set of cultures was secured, at which time every one complained of a slight cold. The teacher expressed the opinion that these colds were chronic and had existed in slight or moderate degree since the epidemic. For this reason this group of cultures has been given the foregoing title.

The bacteriological results have been summarized in Table I and charts I to VII and will be discussed in conjunction with the findings in Labrador.

### *Labrador*

The second study was carried out during July, August, and September at Northwest River, Labrador, a Hudson Bay Company

trading post of about 150 inhabitants, situated at the mouth of Northwest River, where it flows into Lake Melville. The only contact that this community has with the outside world is through Rigolet, 100 miles away on the sea coast, where the mail boat calls about once in three weeks during the summer months. Once during the winter a driver with his dog team brings the mail in over the ice.

The climatic conditions at Northwest River are delightful during the summer months. They resemble most nearly those of Alabama during the month of November. The temperature rarely exceeds 90°F. and only in September did the minimum temperature fall to freezing. The humidity remains consistently low except just before heavy rainfalls.

For purposes of comparison and discussion the people at Northwest River were divided into the following groups.

*Transient.* This group was small (about fifteen) and was composed of summer visitors to Northwest River who came in with the first mail boat at the end of June. The majority of this group were Americans and English who came directly from their home countries. Only "normal" cultures were obtained from this group.

*Permanent.* About ten of this group are whites of American and European extraction who are connected either with the Grenfell Mission station or the Hudson Bay Company and who spent the preceding winter at Northwest River. Only "normal" cultures were secured from this group.

The remainder, and the great majority, of this group is composed of so-called natives. They are a mixture of white and Indian with an occasional trace of Esquimo blood. Their main occupation is trapping, and their economic status is poor. As a result their diet is largely limited to bread, molasses, fish—fresh or salt—and salt meat. Fresh vegetables are practically unknown. The health of the community, despite the pioneer conditions of living, is good. Tuberculosis of the acute fulminating type is the outstanding acute disease. Pneumonia is rare, and when it does occur is mild. In the influenza epidemic of 1918, the mortality was high. A minor epidemic of influenza occurred in March, 1928, with no fatalities. Childhood diseases such as measles, mumps, whooping cough, scarlet fever, and diphtheria are practically unknown.

*Indian.* During the early summer the population of Northwest River was increased by the appearance of about 150 Montaignais Indians, who came to trade with the Hudson Bay Company and receive the ministrations of the Roman Catholic priest. The contact of the Indians with the remainder of the population was slight, and for this reason they have been grouped separately.

The economic status of the Indians is even poorer than that of the natives.

They are a nomadic race living in tents, and their diet is even more restricted than that of the natives. They are subject to about the same diseases as the natives.

*Colds.* Shortly after the arrival of the summer visitors a few cases of colds of moderate severity developed among the native and Indian populations. Only four of the natives were considered as having "bad colds." The Indians spoke no English and visited the laboratory for amusement with a variety of complaints. Thus the classification of "*normal*" and "*colds*" in this group is somewhat confused. Those listed as *normals* were persons with no complaints referable to the respiratory system, but it is difficult to be sure whether or not all in the cold group actually had colds.

*Tracheitis and Contacts.* In the latter part of August, after the departure of the Indians, a definite epidemic of tracheitis developed which affected practically every one in the community in greater or lesser degree, with the exception of the white Grenfell Mission staff and ourselves. Cultures were obtained from the native population only, and were divided into two groups; those with the disease at the time of examination and those listed as "contacts," who were exposed intimately to the disease or who developed it within a few days after the cultures were taken. Because the appearance of this disease was coincident with the arrival of outsiders from a place where so-called influenza was epidemic, and coincident also with the appearance of Pfeiffer bacilli in the community, the bacteriological findings will be discussed when the Pfeiffer bacilli are considered. They are summarized in Table I and Graphs I to VII.

## *Discussion of Findings*

*Groups of Organisms.* The bacteria found have been classified under the headings seen in the table and the graphs. None of these groups requires any special mention except the *intermediates*, composed of Gram-positive diplococci or streptococci, producing green or viridans hemolysis on blood agar plates. The intermediates do not, however, fall definitely either into the green streptococcus or the pneumococcus groups but occupy a midway position, if the classification is based on colony formation, morphology, inulin fermentation, and bile solubility. Since the classification of these organisms is indefinite and since they

are not characteristic pneumococci, they have been recorded separately, and grouped also with the typical green streptococci.

In recording the occurrence of the various types of organisms on the blood agar plates, an estimate of the percentage of each type present was made. The estimates were recorded in multiples of five, and the least estimate was five per cent. The percentage figures for each type of organism in any group of individuals were then added together and divided by the number of examinations made. The resulting figure has been termed the "bacterial group incidence" and represents a figure which would presumably be secured, theoretically at least, by pooling all of the nasopharyngeal swabs from one group of individuals and streaking the pooled emulsion upon one gigantic blood agar plate. Since the persons in each group come into close contact, and their states of health are so similar, the bacterial group incidence figures may be assumed to represent the relative numbers of bacteria present in, as it were, a community throat.

Using this method a figure is obtained, the accuracy of which is undoubtedly open to criticism on technical grounds. When preliminary incubation was used, some groups of organisms were suppressed and others favored, besides which variations in plating technique, and in estimation of percentages of organisms must occur, as well as other technical errors. However, it is believed that these variations and errors are more or less constant and tend to balance one another and that the percentage figure obtained by the method cited above represents a fairly true mean of what may be found in one group of individuals, and is of value in comparing the findings in one group with that in another or the same group in a different state of health, provided that minor differences in figures are disregarded. Therefore in discussing the "bacterial group incidence" only such differences as have been found statistically significant will be considered.

The bacterial group incidence data are imperfect in that they do not show the distribution of organisms in individual throats. Blood agar plates from some individuals in a community showed large numbers of a given organism; others showed few or none. In order to indicate this individual variation we have computed the mean and the standard deviation for each organism and for each series of persons studied. See Table I.

TABLE I

*Bacterial Group Incidence of Nasopharyngeal Flora: Normal, Colds, and Tracheitis*

The mean with standard deviation is given in each instance

Normal

| | No. exam. | Gram neg. cocci % | Staphylo-cocci % | Influenza % | Streptococci % | | | Intermedi-ate % | Green and intermed. % | Pneumo-cocci % | Others % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Hemolytic | Indifferent | Green | | | | |
| Alabama | | | | | | | | | | | |
| 1st Exam. | 17 | 45 ± 6.4 | 2 ± 1.0 | 0 | 0 | 0 | 49 ± 5.7 | 0 | 49 ± 5.7 | 0 | 3 ± 2.0 |
| 2nd " | 17 | 49 ± 8.8 | 2 ± 0.6 | 12 ± 6.3 | 0 | 0 | 24 ± 7.5 | 8 ± 4.9 | 32 ± 7.5 | 0 | 6 ± 2.6 |
| Labrador | | | | | | | | | | | |
| Transient | 19 | 16 ± 6.6 | 2 ± 0.8 | 5 ± 2.4 | 1 ± 1.0 | 12 ± 5.3 | 18 ± 6.4 | 22 ± 8.1 | 40 ± 8.1 | 18 ± 7.9 | 5 ± 3.3 |
| Permanent | 25 | 10 ± 3.2 | 2 ± 0.7 | 2 ± 1.4 | 0 | 13 ± 3.9 | 37 ± 7.2 | 7 ± 4.8 | 44 ± 7.3 | 22 ± 6.5 | 6 ± 2.7 |
| Indian | 21 | 12 ± 2.5 | 2 ± 0.7 | 3 ± 6.9 | 1 ± 1.0 | 11 ± 3.3 | 18 ± 4.6 | 3 ± 2.2 | 21 ± 4.7 | 46 ± 7.3 | 3 ± 1.0 |

Colds

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Alabama-Epidemic | | | | | | | | | | | |
| 1st Exam. | 14 | 15 ± 3.1 | 7 ± 2.5 | 21 ± 5.7 | 6 ± 4.4 | 10 ± 4.6 | 16 ± 5.4 | 19 ± 5.4 | 35 ± 4.0 | 3 ± 1.7 | 3 ± 5.1 |
| 2nd " | 19 | 14 ± 2.4 | 7 ± 2.4 | 18 ± 4.4 | 6 ± 3.0 | 1 ± 1.0 | 30 ± 6.2 | 1 ± 1.0 | 31 ± 6.2 | 19 ± 6.0 | 3 ± 1.7 |
| 3rd " | 15 | 14 ± 4.0 | 3 ± 2.5 | 28 ± 6.1 | 7 ± 3.7 | 0 | 22 ± 5.5 | 23 ± 8.3 | 45 ± 5.4 | 2 ± 1.7 | 1 ± 1.3 |
| Recovery | | | | | | | | | | | |
| 1st Exam. | 15 | 26 ± 5.7 | 2 ± 1.7 | 41 ± 7.7 | 6 ± 3.3 | 4 ± 2.7 | 17 ± 5.1 | 4 ± 2.7 | 21 ± 2.7 | 0 | 0 |
| 2nd " | 19 | 16 ± 5.2 | 7 ± 2.9 | 6 ± 2.5 | 0 | 4 ± 1.7 | 44 ± 7.5 | 12 ± 6.3 | 56 ± 7.3 | 1 ± 0.5 | — |
| Labrador Sporadic | | | | | | | | | | | |
| Permanent | 23 | 21 ± 5.3 | 2 ± 1.0 | 5 ± 3.0 | 0 | 15 ± 4.6 | 16 ± 4.2 | 10 ± 4.5 | 26 ± 5.5 | 23 ± 6.9 | 8 ± 3.9 |
| Indian | 19 | 12 ± 2.5 | 4 ± 1.3 | 3 ± 2.6 | 0 | 3 ± 1.4 | 19 ± 6.6 | 6 ± 4.4 | 25 ± 3.3 | 45 ± 6.5 | 9 ± 3.5 |

Tracheitis

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Labrador | | | | | | | | | | | |
| Contacts | 16 | 15 ± 4.8 | 4 ± 3.7 | 20 ± 7.5 | 0 | 3 ± 1.7 | 17 ± 6.9 | 14 ± 6.5 | 31 ± 7.7 | 21 ± 7.2 | 5 ± 3.1 |
| Tracheitis | 35 | 15 ± 2.6 | 3 ± 2.6 | 12 ± 3.7 | 3 ± 1.0 | 17 ± 4.1 | 22 ± 4.4 | 10 ± 3.5 | 32 ± 5.1 | 16 ± 3.3 | 3 ± 1 |

The incidence of any given organism in individual throats is shown in the graphs. For example, in Graph I—Gram-negative cocci—we have indicated the total percentage of persons in each series harboring this type of organism. We then subdivided the positive cases into four groups on the basis of the proportional number of this particular type of organism found on the individual blood agar plates. Thus in the series of Alabama normals, on first examination, 100% of the persons showed gram-negative cocci, over one fourth (27%) of these persons had from 1–25% of gram-negative cocci in relation to all other organisms, 37 per cent of the persons showed 26 to 50% gram-negative cocci, 22% showed 51 to 75% of these organisms, and in the remaining 14% of the positive cases from 75 to 100% of the total flora were gram-negative cocci. See Graph I.

The findings for the "staphylococci," "hemolytic streptococci," and "others" have not been represented graphically because of the relatively small numbers of these organisms found.

During the course of this study various individuals have been examined for the presence of filter-passing anaerobic organisms. The results will be presented in another report.

*Gram-Negative Cocci.* (Table I, Chart I). During the study considerable variation in colony formation, pigment production, and action on sugar media of the same strains of organisms in this group have been noted. Recently Wilson and Smith (2) have reported at length on such variations and concluded that the members vary from time to time, and they suggest that for the present, at least, the members, with the exception of the meningococci, be classified as belonging to a large variable group and no attempt be made to divide them into species on the basis of the above-mentioned factors. For this reason we have made no effort to divide the members of this type into species. We found no meningococci.

The bacterial group incidence is fairly constant in all of the persons studied except the Alabama normals. The relatively large numbers of gram-negative cocci present in the nasopharynx of the normal persons studied in Alabama may be due to the fact that blood agar plates were inoculated directly with the swabs without giving them a preliminary blood broth incubation. The individual incidence is about the same in all the persons studied. During the periods of acute infection

there is a tendency to suppression of the organisms. This fact suggests that the Gram-negative cocci play no part in the acute respiratory infections.

*Staphylococci.* (Table I). The types found were *S. albus* and *S. aureus* and in Labrador an occasional *S. citreus*. A few of the *S. albus* and *S. aureus* strains were hemolytic.

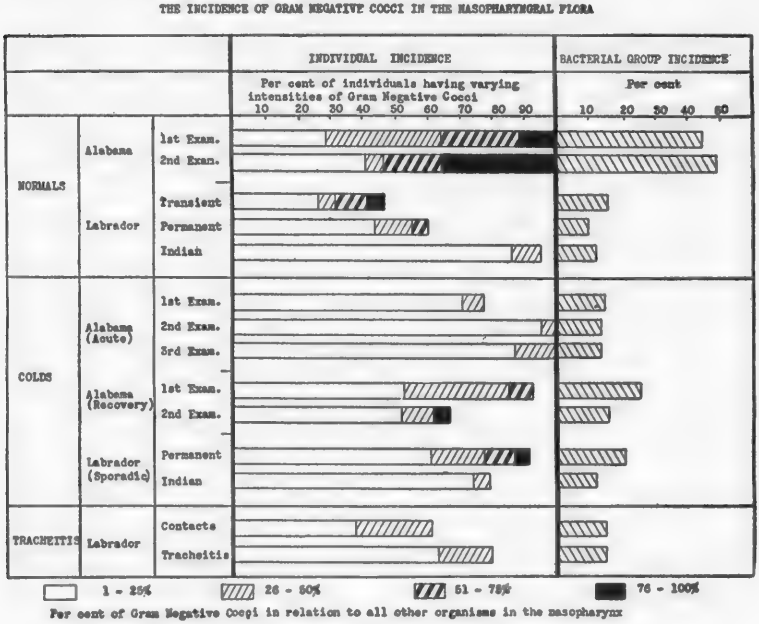


CHART I

The bacterial group incidence of these organisms is low, in normal persons small numbers being found in about half of the individuals examined. During infection the incidence rose slightly, though there was not a constant distribution. A few individuals harbored large numbers and in the remainder staphylococci were entirely absent. During the recovery period in Alabama, almost pure cultures of staphlococci were recovered from certain individuals with chronic colds.

*Pfeiffer Bacilli.* (Table I, Chart II). This group of organisms is

composed of Gram-negative rods requiring some accessory factors for growth and includes the para and hemolytic strains.

The bacterial group incidence in Alabama increased with the appearance of the cold epidemic as it did in Labrador with the appearance of the tracheitis epidemic. In Alabama, however, the highest incidence was found one month after the epidemic had reached its peak. In Labrador it was not possible to determine whether or not the

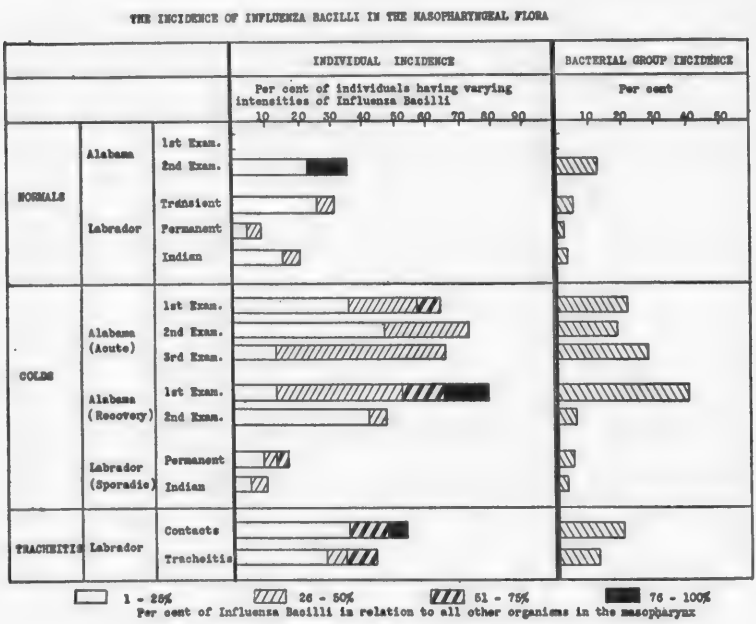


CHART II

highest incidence occurred sometime after the epidemic abated, because of the discontinuance of this study just at the end of the epidemic. In general, the individual incidence rose parallel with the bacterial group incidence.

During normal periods, particularly in Labrador, the majority of the Pfeiffer strains which were recovered were either para or hemolytic in type. During periods of infection there appeared large

numbers of true Pfeiffer bacilli, e.g. non-hemolytic strains requiring both v and x substance for their growth. Most of them were of the indol-forming type. The numbers of para strains recovered remained about constant at all times, while during epidemics there was a marked increase in the number of strains of true Pfeiffer bacilli recovered. This phenomenon was noted by Park (3) and his co-workers.

In Labrador the association of Pfeiffer bacilli with the outbreak of the tracheitis epidemic was so conspicuous that the following detailed story of the epidemic is included at this point.

During the latter part of July an epidemic of acute respiratory disease appeared in Cartwright and other coastal settlements over one hundred miles away. The cases were characterized by acute onset, prostration, bone aches, and fever of about five days' duration. Many patients remained prostrated for two or three weeks after other symptoms disappeared. The cases were diagnosed as "influenza" by the three physicians of the Mission hospitals on the coast.

The mail boat arrived in Northwest River from the coast on August 8, bringing from Cartwright a child of nine years, who probably had just recovered from an attack of "influenza." Her contacts after arrival in Northwest River were few, and apparently no secondary case occurred from contact with this child. On August 9, two natives returned from a trip to the coast. One had caught a slight cold in Indian Harbor following association with deep sea fishermen. The man with the cold lived on the south bank of Northwest River and had little contact with the community. The day following his return, his wife had a chill and headache and came to the laboratory on the 15th complaining of a cold. Her only symptom was sore throat, and cultures showed no Pfeiffer bacilli.

On August 15 an English clergyman arrived from Cartwright and brought the first news of the influenza epidemic. He had been closely associated with it but had not been ill. Cultures of his nasopharynx taken one hour after arrival showed about ten per cent Pfeiffer bacilli. Otherwise, the flora was similar to that of the local inhabitants. During the next two days he visited all of the houses in the village and on August 16 held a communion service which was attended by practically every one in the village.

The results assembled under "contacts" were obtained during the next few days.

On August 18 the native who returned in good health from Indian Harbor awoke with a headache and a tickling senation in the throat, localized by him as just below the larynx, and accompanied by a dry unproductive cough. Two days later his wife developed an illness with the same onset. The infection spread rapidly, and by September 4 practically every one in the village had had a similar attack. Individuals reacted differently, but general tendencies were noticeable. In children, the irritation and cough continued for several weeks and were much

worse at night. The younger adults who had the attack early in the outbreak recovered rather promptly with few or no other symptoms. Those who were attacked later in the outbreak had the usual onset, but two or three days later had what may be best described as violent head colds with prostration and fever of several days' duration. The older adults were entirely unaffected. Upon our departure on September 17 numerous cases of the head colds persisted, but in general the epidemic could be said to be subsiding.

In summarizing the facts of this outbreak it can be said that following the arrival of a potential carrier or carriers of "influenza organisms" directly or indirectly from Cartwright, where there had been an influenza epidemic, there occurred an epidemic of tracheitis at Northwest River. The most probable carrier of the organism was the clergyman who harbored Pfeiffer bacilli in his nasopharynx on arrival. Coincident with the epidemic, (in Northwest River), there was a statistically significant increase in the bacterial group incidence and the individual incidence of true Pfeiffer bacilli in the community. See Chart II and Table I.)

*Hemolytic Streptococci.* The types of organisms found in Alabama were *St. pyogenes*, *St. anginosus*, and *St. infrequens* (according to Holman's classification). The only type found in Labrador was *St. pyogenes*, of a low virulence for mice.

Few persons harbored members of this group and in these persons the numbers of organisms present were small. In Alabama these organisms were found only during the cold epidemic, and in Labrador, with one exception, only at the time of the tracheitis epidemic. In Labrador the streptococci were found in the nasopharynx of individuals who had impetigo, from the lesions of which similar strains of *St. pyogenes* of low virulence were recovered. These persons were all examined one or more times before the onset of the epidemic, and the hemolytic streptococci were not found in the nasopharynx until after the outbreak. These findings suggest that the hemolytic streptococci play the rôle of a secondary invader and resemble in this respect the staphylococci.

*Indifferent Streptococci.* (Table I, Chart III). In this group are included Gram-positive cocci, usually in short chains with a characteristic colony formation, that do not act upon blood and that almost invariably ferment inulin. Upon lactose and salicin the action is variable.

This type of organism was not found during the normal period in Alabama. Early in the cold epidemic a few strains were found in an occasional individual and were then present in small numbers only. In Labrador, on the contrary, the organisms were present in moderate numbers and in the great majority of persons examined and constituted apparently part of the normal flora.

A small series of observations made upon the members of the transient group of persons at Northwest River indicates that the indifferent

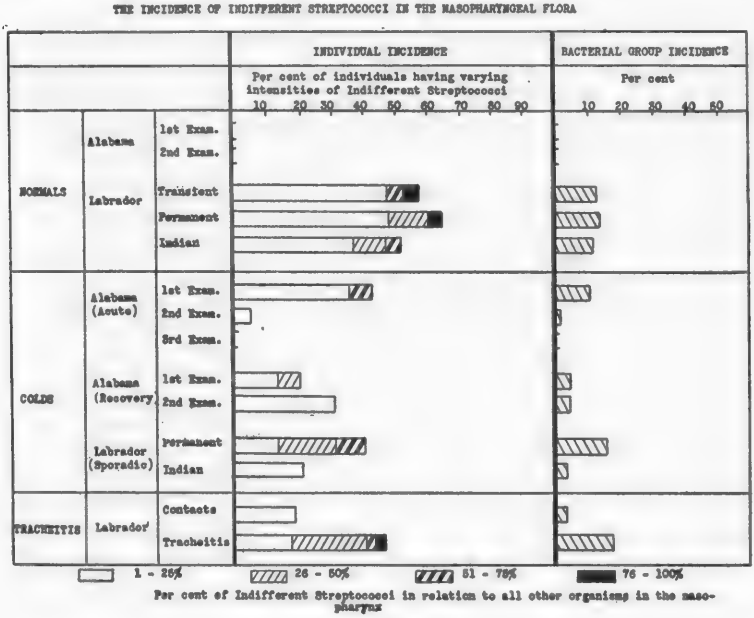


CHART III

streptococcus was implanted in these persons after their arrival at Northwest River, and became a part of their normal flora. These persons were examined ten days after their arrival and showed no indifferent streptococci. Second and third examinations of these individuals were made later during the summer and with each examination increasing numbers of indifferent streptococci were recovered.

*Green Streptococci, Intermediates, and Pneumococci.* (Table I,

Charts IV, V, VI, VII). Because of the cultural relationships of these organisms they will be discussed together. The types of green streptococci and the order of frequency with which they were found were *St. salivarius*, *St. mitis*, *St. equinus*, *St. ignavus* and *St. fecalis*. The last was found rarely. *St. salivarius* was the most common while *St. equinus* and *St. ignavus* were found occasionally in each group.

The "intermediates" were organisms which resemble both pneumococci and *St. viridans*. Their fermentation reactions are, as a rule,

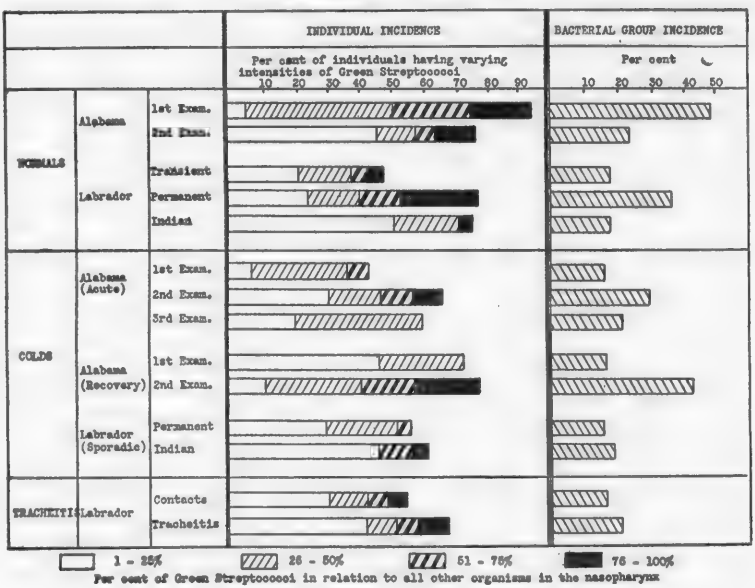


CHART IV

like those of *St. salivarius*, but in addition they fermented inulin. The colony formation and the morphology in smears was such as to suggest the pneumococcus. These strains were usually partially bile soluble. This group also includes strains that were typical green streptococci except that they were bile soluble. The outstanding type of this group showed characteristic green colonies and morphology, did not ferment inulin, and in broth grew in a mass at the bottom of the tube.

This mass when broken up did not emulsify smoothly, but formed large flakes which were bile soluble. The members of this group that were tested were not virulent for mice.

The organisms classified as pneumococci were typical in colony formation, morphology, inulin fermentation, and bile solubility. In Alabama, virulence tests were not made, but in Labrador a sufficient number were carried out to show that the organisms were comparatively avirulent to white mice.

THE INCIDENCE OF INTERMEDIATES IN THE NASOPHARYNGEAL FLORA

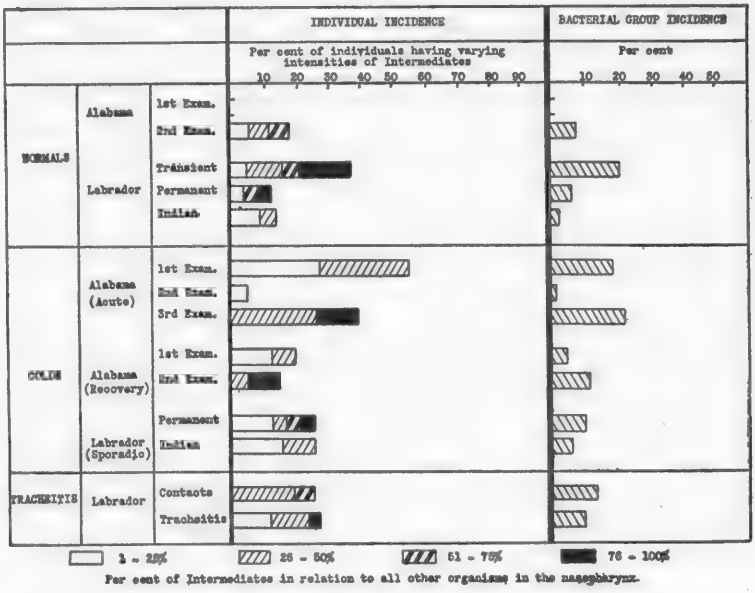


CHART V

The green streptococci were obviously normal inhabitants of the nasopharynx. With the onset of an "infection" there was a diminution in numbers in the affected person with complete suppression in a few individuals. (See Chart IV.)

The intermediates were found in all groups, and in general, as they appeared, the green streptococci diminished. Whether this reciprocal

relation is due to errors in detecting colonies for identification and in estimating the numbers present, or whether the disappearance of the green streptococci is accompanied by a definite increase in the intermediates, was not determined definitely.

Examinations during the normal period in Alabama gave few or no intermediates. When the cold epidemic first appeared in Happy Hollow school the green streptococci were suppressed and the inter-

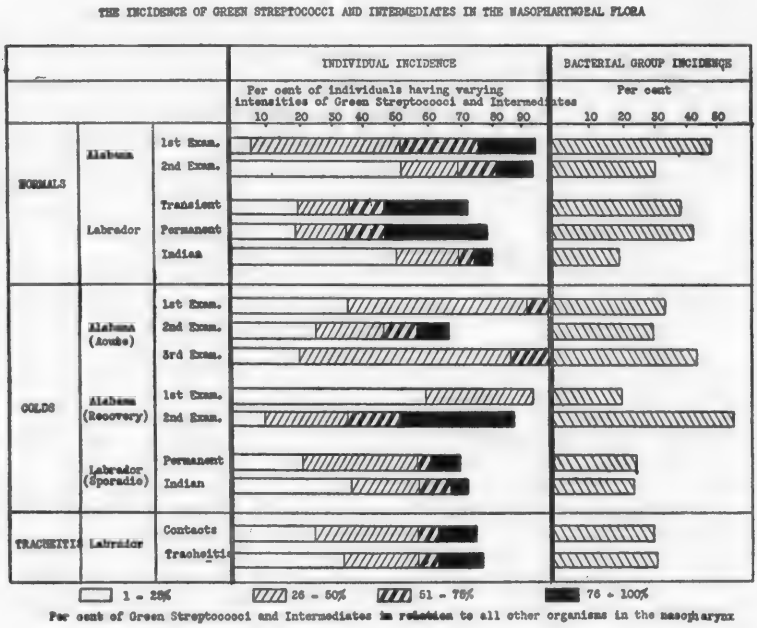


CHART VI

mediates appeared in numbers equivalent to the missing green streptococci. At the height of the epidemic, the intermediates had disappeared while the green streptococci returned to approximately their previous numbers. Coincident with the disappearance of the intermediates there was a significant rise in the number of pneumococci found.

*Pneumococcus.* (See Chart VII.) In Alabama the pneumococcus

was not found during the normal period. Coincident with the appearance of the epidemic of colds, pneumococci were found in considerable numbers, the incidence being highest at the height of the epidemic. At the end of the epidemic the pneumococci disappeared.

In Labrador, on the other hand, pneumococci made up a large part of the normal flora. The transient population showed the lowest incidence, the trappers a relatively high incidence, and the Indians the

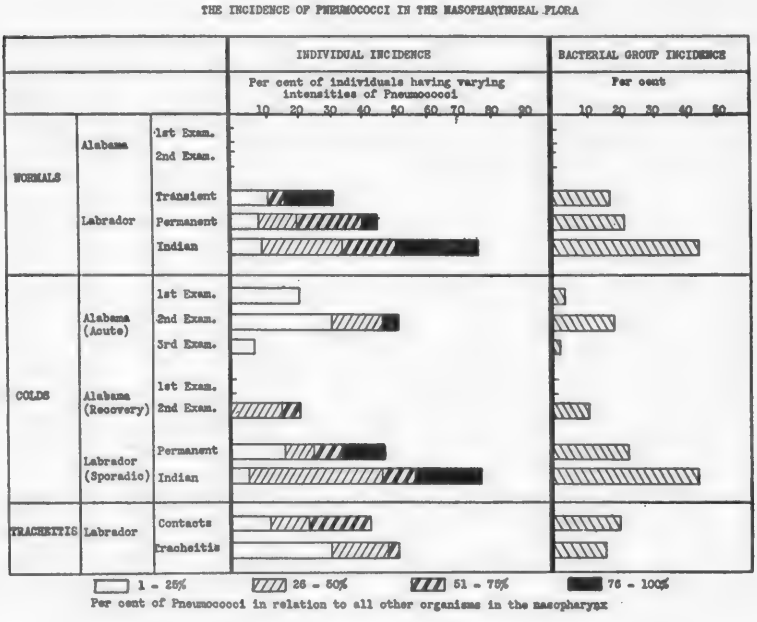


CHART VII

highest of all. In fact, the predominating organism in the throats of the normal Indians was a pneumococcus, type IV, of low virulence to mice. No colds occurred among the transient group, but in those individuals from both Indian and permanent groups who had sporadic colds there occurred no increase in the incidence of pneumococci. The epidemic of tracheitis, which affected the permanent population after the nomadic Indians had left the Post, was not accompanied by an

increase in pneumococci. If the Indians are considered as the group furthest removed from civilization it follows that the pneumococcus becomes increasingly a part of the normal flora in Labrador as civilization is left behind. Yet pneumonia is rare in these regions both among the trappers and the Indians and when it occurs it is seldom fatal.

*Other Organisms.* In this classification we have included a miscellany of types of organisms, none of which was found with any regularity or associated with any pathological condition. The outstanding forms were diphtheroids, large Gram-negative rods, and strains of small Gram-positive cocci which produced no, or slight, traces of viridans hemolysis when grown in mass cultures on blood agar plates. Some of the latter strains slightly fermented dextrose, others fermented no sugar whatever. In colony formation these cocci resembled the indifferent streptococci and would have been so classified but for the traces of hemolysis and their inactivity in sugar media.

### *Comparison of Findings with the Results of Other Investigations*

In comparing the results of the present investigation with those obtained by others, several rather well-established facts have been confirmed. The Gram-negative cocci, staphylococci, green streptococci, diphtheroids, and possibly also hemolytic streptococci are normal inhabitants of the nasopharynx. In so far as statistical methods offer any proof, none of these groups of organisms was related to the two epidemics of colds which were studied.

The method of reporting results adopted by Park (3) and his co-workers is essentially the one used in the present study and although the technique used by him was more elaborate the results can be compared directly. The general agreement in results leads to the belief that the discrepancies noted are real and not dependent upon variations in technique. Table II compares our results with those of Park and his co-workers.

The chief differences between the New York data as given by Park and those from Alabama and Labrador are:

*Normal Flora.* Pneumococci were more prevalent in New York than in Alabama, whereas in Labrador pneumococci were much more prevalent than in New York. Pfeiffer bacilli were more prevalent in

TABLE II

*Comparison of Nasopharyngeal Flora in New York (Park et al.) with Alabama and Labrador*

| | | Gram-neg. cocci | Staphylococci | Pfeiffer bacilli | Hemolytic strep. | Indifferent strep. | Green Strep. & Intermediates | Pneumococci | Others | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| **Normal** | | | | | | | | | | |
| Bacterial Group Incidence | Alabama | 47 | 2 | 6 | 0 | 0 | 40 | 0 | 5 | Average of two normal examinations. |
| | Labrador | 11 | 2 | 2 | 1 | 12 | 33 | 34 | 5 | Average of Indian and Permanent group. |
| | New York | 29 | 6 | 9 | 1 | 2 | 32 | 8 | 11 | |
| Individual Incidence | Alabama | 100 | 24 | 17 | 0 | 0 | 95 | 0 | 18 | |
| | Labrador | 89 | 33 | 14 | 5 | 58 | 80 | 60 | 33 | |
| | New York | 87 | 12 | 40 | 6 | 4 | 89 | 26 | 39 | |
| **Colds** | | | | | | | | | | |
| Bacterial Group Incidence | Alabama | 14 | 7 | 18 | 6 | 1 | 31 | 19 | 3 | Height of epidemic. |
| | Labrador (Sporadic colds) | 17 | 3 | 4 | 0 | 9 | 26 | 34 | 7 | Average of Indian and Permanent. |
| | Labrador (Tracheitis) | 15 | 3 | 15 | 2 | 12 | 32 | 18 | 4 | Average of Contacts and Tracheitis. |
| | New York | 19 | 2 | 17 | 3 | 2 | 33 | 9 | 19 | |
| Individual Incidence | Alabama | 100 | 20— | 75 | 10— | 5 | 65 | 52 | 25— | |
| | Labrador (Sporadic colds) | 87 | 20— | 12 | 10— | 30 | 65 | 63 | | |
| | Labrador (Tracheitis) | 73 | 20— | 45 | 1— | 55 | 78 | 49 | 25— | |
| | New York | 87 | 65 | 65 | 16 | 34 | 91 | 39 | 70 | |

New York than in Alabama or Labrador. Indifferent streptococci were more prevalent in Labrador than in New York. In Alabama these organisms were not found in normal throats. Hemolytic streptococci were not as prevalent in normal throats in Labrador or Alabama as in New York.

*Colds and Tracheitis.* An epidemic of colds in Alabama was accompanied by an increase in pneumococci and Pfeiffer bacilli. Sporadic colds in Labrador did not change the essential picture but an epidemic of tracheitis was accompnaied by an increase in the prevalence of Pfeiffer bacilli. In New York colds were accompanied by an increase in Pfeiffer bacilli.

Except for these rather striking differences, there is close parallelism between the nasopharyngeal flora in an isolated semitropical farming community in Alabama, a remote semi-arctic trading post in Labrador, and New York City.

## SUMMARY

Studies of the bacterial flora of the nasopharynx were made in isolated communities in South Alabama and Labrador. The basic flora was determined in both communities. In Alabama an epidemic of common colds was studied. In Labrador cases of sporadic colds and an epidemic of tracheitis were studied.

Gram-negative cocci were found in nearly all normal individuals in moderate numbers. In pathological states there was a suppression of these organisms.

Staphylococci were found in small numbers in about half of the normal individuals. In pathological conditions they disappeared from most of those affected but were found in increased numbers in a few individuals.

Pfeiffer bacilli were absent or present only in small numbers in normal individuals. During the epidemic of colds in Alabama there was an increase in the number of strains recovered and an increase in the relative numbers of the bacilli in each throat. The highest prevalence was found one month after the epidemic had reached its height. In Labrador a similar increase was coincident with an epidemic of tracheitis. During normal periods the majority of the Pfeiffer strains were of the para non-indol-forming type. During

epidemic periods the strains recovered were largely true indol-forming *B. Pfeifferi.*

Hemolytic streptococci were rarely found in normals. During disease prevalence periods they appeared in a small number of persons.

In Alabama, indifferent streptococci resembled the hemolytic streptococci in their distribution. In Labrador they were found to be widely distributed in both health and disease and composed apparently a part of the normal flora.

Green streptococci were found to be widely distributed in fairly large numbers both in health and disease.

Intermediates, or organisms midway between green streptococci and pneumococci, were found in moderate numbers in each series of persons studied. Early in the Alabama epidemic they were present in large numbers in nearly all persons.

Pneumococci were not found in Alabama in normal individuals. The epidemic of colds in Alabama was accompanied by a marked increase in the incidence of these organisms. In Labrador pneumococci seemed to be part of the normal flora as they were generally distributed throughout the community, in many instances comprising a large proportion of the flora of an individual's throat. The Labrador strains of pneumococci were avirulent.

A variety of other organisms such as diphtheroids, Gram-negative rods, and Gram-positive cocci were found in small numbers in many individuals both in health and disease.

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# DEVELOPMENT IN TISSUE CULTURES OF THE INTRACELLULAR CHANGES CHARACTERISTIC OF VACCINAL AND HERPETIC INFECTIONS

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Plates 29 to 31

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In a preliminary note (1)* a method of obtaining in tissue cultures intracellular changes characteristic of vaccinal and herpetic infections was briefly described. The purpose of the present paper is to give a detailed report concerning this method and some of the results secured through its use.

In 1906, Aldershoff and Broers (3), in order to prove that Guarnieri bodies are not derived from leucocytes, inoculated rabbit corneas with vaccine virus, immediately removed them from the animals, and incubated them in sterile serum at 37°C. This method was unsuccessful, the corneas becoming contaminated with bacteria which in a liquid medium interfered with the work. Thereupon the workers altered their procedure and placed the inoculated corneas, suspended in physiological salt solution, in humid chambers. After incubation for different periods of time, the tissues were fixed, sectioned, stained, and examined for the presence of vaccine bodies. Guarnieri bodies were found in corneas treated in this manner.

Gins (4), in 1916, using the coverslip method for the cultivation of vaccine virus in corneal cells, obtained what he considered Guarnieri bodies. Later (5), however, he stated that the bodies observed by him in 1916 were probably not vaccine bodies.

The majority of workers, including Steinhardt, Israeli, and Lambert (6), Harde (7), and Haagen (8), who searched for Guarnieri bodies in their tissue

* Shortly after this note was sent in for publication, one of the authors (Rivers) received a letter from Dr. C. H. Andrewes regarding the appearance of inclusions in cultures of testicular tissue infected with Virus III. His observations and our findings were made independently and approximately simultaneously. A preliminary note (2) of his work appeared in *Brit. Jour. Exp. Path.*, 1929, **10**, 188.

cultures infected with vaccine virus, failed to find them. It also appears that no investigator has observed nuclear inclusions in tissue cultures infected with herpetic virus. Andrewes (2), however, in a recent report, has described the appearance of characteristic nuclear changes in testicular tissue infected with Virus III and cultivated *in vitro*.

Intracellular changes, "inclusion bodies," either cytoplasmic or nuclear, are characteristically associated with certain virus infections as they occur within the living host. These alterations, although their exact nature has not as yet been determined, are frequently used for diagnostic purposes. In spite of the fact that inclusion bodies occur in infected cells within the host, no observer, until recently (1, 2) has found them in tissues infected and grown *in vitro*. Inasmuch as virus infections, virus immunity, and even the nature of viruses themselves are poorly understood, it seemed that, if it were possible to devise a means of securing visible evidences of virus activity in tissue cultures, definite information might be found concerning many obscure points in this field of work. After a number of unsuccessful attempts, the following method of regularly obtaining Guarnieri bodies and herpetic nuclear inclusions in tissue cultures was evolved.

## *Materials*

*Viruses.*—Vaccine virus and the virus of herpes simplex were employed because the rabbit is equally susceptible to both. Furthermore, the vaccine virus produces cytoplasmic changes while that of herpes induces nuclear alterations. Thus, one virus served as a control on the other.

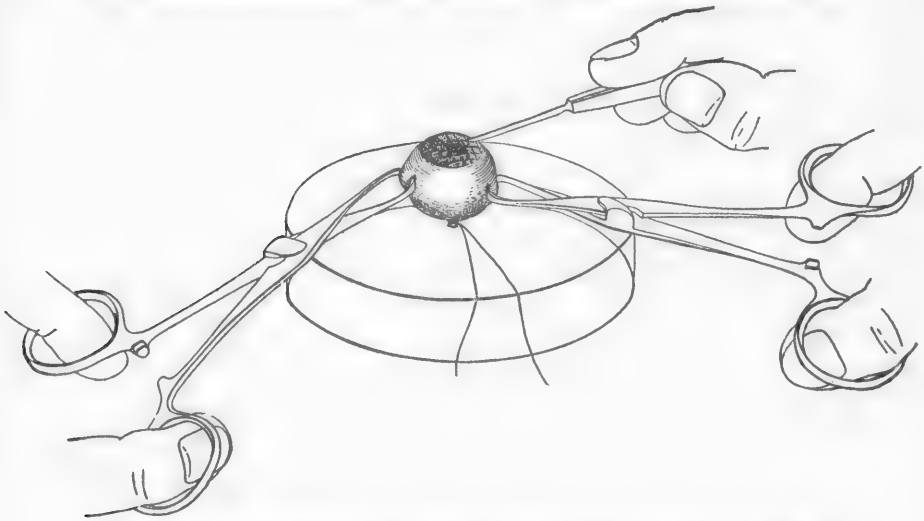
Levaditi's neurovaccine propagated in the testicles of rabbits, and Noguchi's testicular virus, were the strains of vaccine virus used. The herpetic virus chosen for the work was the H. F. strain isolated by Flexner and Amoss. In all experiments save one, freshly prepared virus emulsions without glycerol were employed. For constant results, one must conduct the experiments with fresh potent virus to which no preservative has been added.

*Tissues.*—For this work, rabbit corneal tissue was chosen because of the following facts: 1) rabbit corneal epithelium is equally susceptible to the viruses of vaccinia and herpes simplex; 2) Guarnieri bodies and herpetic inclusion in cells of this tissue have been extensively investigated; 3) in the past, corneal cells have frequently been used for the *in vitro* cultivation of vaccine virus; 4) many studies concerning vaccinal and herpetic immunity within the animal have been conducted in the cornea of rabbits.

*Procedure*

A rabbit, under light ether anaesthesia, was exsanguinated from the heart by means of a syringe into which 1 cc. of a 1–1,000 heparin solution for each 10 cc. of blood had been previously drawn. Then, to obtain a tissue extract, the spleen was excised, minced, added to a small amount of Ringer's solution, and centrifuged. Finally, both eyes were enucleated intact, after stout silk ligatures had been placed around the nerve and large vessels.

The eyes, fixed in the grip of special forceps (Text-fig. 1) and suspended over a large Petri dish, were thoroughly washed with Ringer's solution to remove as many contaminating bacteria as was possible. With a sterile cataract knife the



TEXT-FIG. 1. Manner in which corneas were scarified and infected with the viruses.

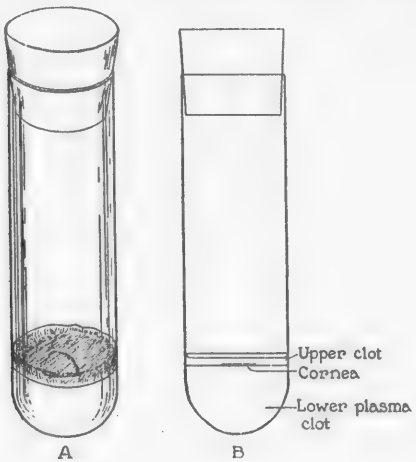
eyes to be used as controls were cross-hatched (Text-fig. 1) with closely spaced scarifications just deep enough to penetrate the epithelial layer. Test eyes were treated in a similar manner with the exception that prior to each scarifying stroke the knife was dipped in a virus emulsion. The cornea was then removed from each eye and divided into 4 to 8 pieces. The pieces from test eyes were further inoculated by immersion in an emulsion of virus for 1 to 3 hours at 37°C.

In some experiments antemortem inoculations were made. Following an instillation of a 2 per cent cocain solution, the corneas were scarified and inoculated in the usual manner. 1 or 2 hours later the animals were sacrificed, the eyes were enucleated and washed with Ringer's solution, and the corneas were removed and divided into 4 to 8 pieces.

With the plasma, spleen extract, and bits of cornea, cultures were set up. Into a sterile 50 cc. pyrex centrifuge tube 2 or 3 cc. of plasma were introduced, to

which a few drops of tissue extract were added to induce clotting. On top of each clot was placed 1 piece of normal or infected cornea which was then covered with another thin clot of plasma (1 cc.) and spleen extract (Text-fig. 2). The tubes, sealed with sterile corks, were placed in an incubator at 37°C. for 24, 48, and 72 hours.

After incubation the clots, containing their bits of cornea, were removed from the tubes, fixed in Zenker's fluid, embedded in paraffin, sectioned, and stained according to Giemsa's method or with eosin and methylene blue. Phloxine at times was substituted for eosin. Then many sections from each block of tissue were examined for the presence of characteristic inclusions. As controls for the




TEXT-FIG. 2. Appearance of cultures prepared in 50 cc. pyrex centrifuge tubes.

presence of active virus in the cultures, preparations similar to those studied histologically were emulsified and tested in the skin of rabbits.

## RESULTS

Numerous observations have been made on the virus of vaccinia, and 2 sets of experiments have been performed with herpetic virus. Inasmuch as corneal tissue from adult rabbits was used, one might suppose that contaminating bacteria would have interfered with the work. Many of the tubes, however, showed no evidence of bacterial contaminations; others contained only a few colonies of organisms that did not disturb the activity of the viruses; and a few were

sufficiently infected with bacteria to be valueless. To circumvent the invalidation of results by bacterial contaminants, experiments were always set up in duplicate and occasionally in triplicate.

In this work Levaditi's strain of neurovaccine produced satisfactory lesions more consistently than did Noguchi's testicular virus. Although on one occasion positive results were obtained with a glycerolated virus, it seemed advisable to use a freshly prepared virus emulsion for each experiment. Corneas infected *in vivo* and *in vitro* showed excellent lesions regularly. The latter, however, were less frequently contaminated.

In each experiment, bits of cornea were fixed for examination after 24 and 48 hours of incubation. Characteristic lesions with inclusion bodies were found in infected tissues after both periods of incubation. If the virus were very potent, however, and if the inoculation were highly successful, 24 hours post-inoculation was found to be a suitable time for examination of the tissues, otherwise 48 hours proved to be more favorable. Some preparations were allowed to remain at 37°C. for 3 days. In these the infected epithelium was extensively disintegrated and unsuitable for histological examination.

Control tissues always revealed definite evidence of growth of epithelial cells in the form of new epithelium filling in the defects caused by scarification (Fig. 13). At times mitotic figures were observed (Fig. 7). Frequently the tissues appeared to be almost as normal as those treated in a similar manner with the exception of being allowed to remain in the animal. If the inoculations were made *in vitro,* the absence of leucocytes was striking. Significant inclusions were never seen in the controls.

Corneas inoculated with vaccine virus showed either discrete lesions separated by normal tissue or a diffuse infection involving all the epithelium. At the sites of infection the epithelium was thickened (Figs. 3, 6, 11, 14) because of an increase both in the number of cells and in the size of individual cells. Numerous typical Guarnieri bodies (Figs. 8, 9, 11, 12, 14) situated in clear spaces within the cytoplasm of epithelial cells were found. These intracellular changes occurred both in the cells present at the time of inoculation and in the new cells (Fig. 12) growing in to fill the defects produced in the corneal epithelium by scarification. On several occasions cells undergoing

mitosis were observed to contain vaccine bodies (Fig. 9). In addition to the Guarnieri bodies, smaller, irregular, basophilic structures that were considered to be Paschen bodies at times studded the cytoplasm of many cells. The end result of the vaccinal infection as studied under the conditions of these experiments was a complete dissolution of the majority of the involved cells (Fig. 3). By testing the material on the skin of normal rabbits, active vaccine virus was demonstrated in the cultures exhibiting characteristic lesions.

The herpetic virus produced striking changes in the infected corneas. In certain areas (Figs. 4, 15) the increase in the thickness of the epithelium produced hummocks. The intracellular bridges disappeared, the cells were swollen, many amitotic giant cells appeared, and numerous typical acidophilic nuclear inclusions were present (Fig. 15). Structures resembling Guarnieri bodies were never seen.

## DISCUSSION

The *in vitro* production of pathological pictures similar to those caused *in vivo* by infectious agents has rarely been accomplished. Maximow (9, 10) and Lang (11), however, have reported that certain tissues cultivated and infected *in vitro* with tubercle bacilli evince pathological changes resembling those observed in infected hosts. In spite of the fact that the viruses of vaccinia and herpes simplex can be cultivated in tissue cultures, no one has been able previously to show definitely that characteristic cytologic lesions of these diseases occur in such cultures. Therefore, the method described above is the first one evolved by which typical vaccinal and herpetic lesions may be regularly produced in tissues inoculated and cultivated *in vitro*. Rabbit corneas handled in a special manner were used for this purpose. In view of Andrewe's (2) work, it seems that other viruses and other tissues may be employed for similar studies. The paper that immediately follows indicates that such methods offer a new means of studying and analyzing virus infections and virus immunity.

## SUMMARY

Characteristic vaccinal and herpetic lesions, including Guarnieri bodies and acidophilic nuclear inclusions respectively, regularly occur in rabbit corneas infected and cultivated *in vitro* according to the method here described.

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## EXPLANATION OF PLATES

### PLATE 29

FIG. 1. Section of a cornea immediately after *in vitro* scarification and inoculation. Note the breaks in the epithelial cells induced by scarification. Giemsa. × 155.

FIG. 2. Section of a bit of normal cornea after cultivation for 48 hours in normal plasma and spleen extract. Eosin and methylene blue. × 155.

FIG. 3. Section of a 48-hour vaccinal lesion in a cornea infected and cultivated *in vitro*. Note thickening of the layer of epithelial cells which are beginning to disintegrate. Compare with Figs. 1 and 2. Eosin and methylene blue. × 155.

FIG. 4. Section of a 48-hour herpetic lesion in a cornea infected and cultivated *in vitro*. Giemsa. × 155.

FIG. 5. Corneal epithelial cells 2 hours after inoculation with vaccine virus. Compare with Fig. 6. Eosin and methylene blue. × 380.

FIG. 6. Corneal epithelial cells 48 hours after *in vitro* inoculation with vaccine virus and cultivation in normal plasma. Compare with Fig. 5. Eosin and methylene blue. × 380.

FIG. 7. Mitotic figure in a 48-hour corneal culture. Giemsa. × 1,000.

FIG. 8. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies and mitotic figure. Giemsa. × 1,000.

FIG. 9. Section of a 48-hour corneal culture infected with vaccine virus. Note Guarnieri bodies, cell with mitotic figure and Guarnieri body, and beginning dissolution of the epithelial cells. Giemsa. × 1,000.

PLATE 30

FIG. 10. Section of a cornea infected (2 hours) with vaccine virus *in vivo*. Note the defect caused by scarification. A bit of the cornea was fixed as a control immediately after removal from the rabbit. Compare with Figs. 11 and 12. Giemsa. × 380.

FIG. 11. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated *in vitro* for 48 hours. Note the difference in thickness exhibited by the layers of epithelium in the two figures. Insert (× 1,000) shows a Guarnieri body in one epithelial cell engulfed by another. Giemsa. × 380.

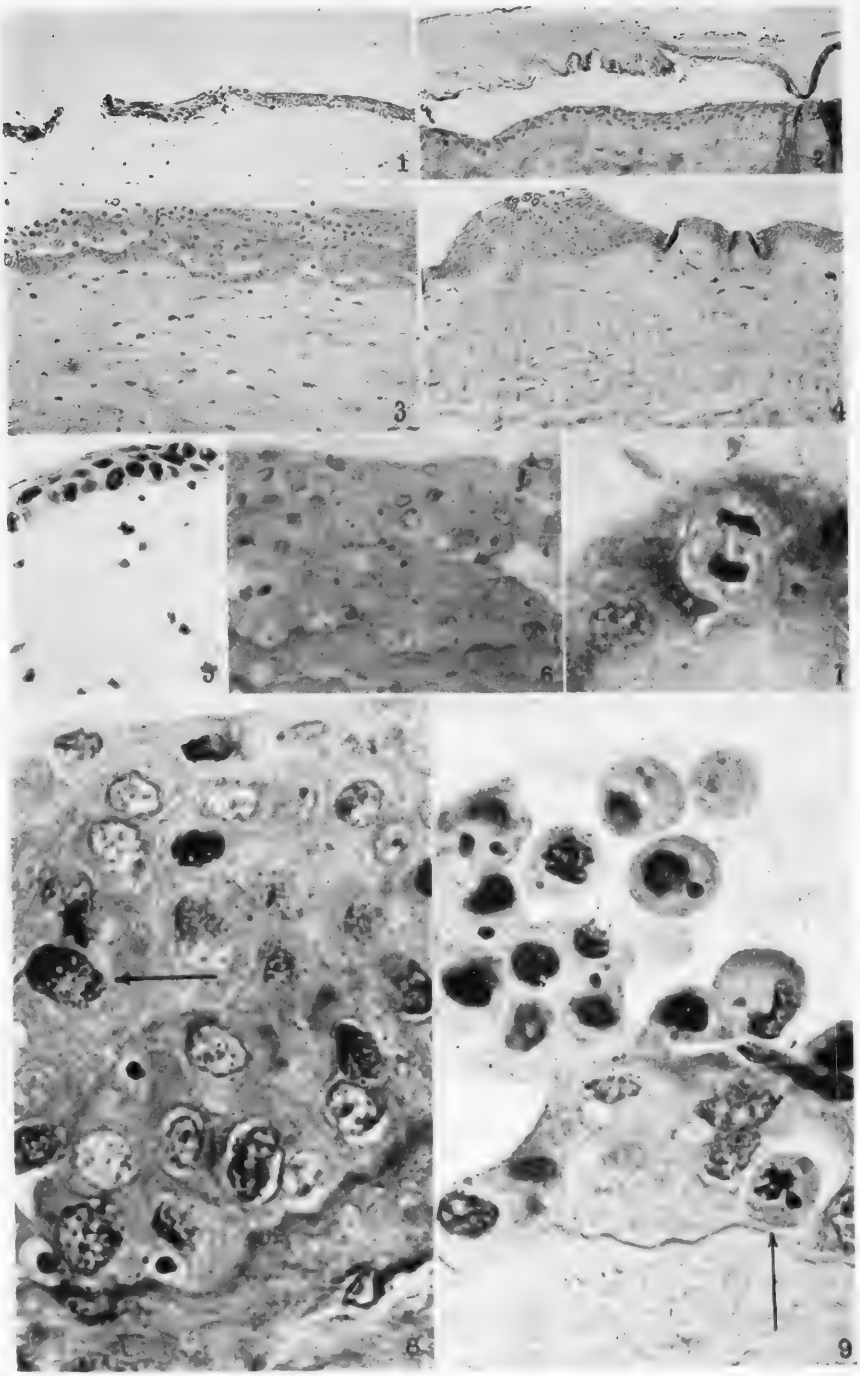
FIG. 12. Section of a piece of cornea similar to that shown in Fig. 10, with the exception that it was cultivated *in vitro* for 48 hours. Note that the defect in the layer of epithelial cells has been filled in with new cells, many of which contain Guarnieri bodies. Insert (× 1,000) shows a Guarnieri body in one cell engulfed by another. Giemsa. × 700.

PLATE 31

FIG. 13. Section of a bit of normal cornea cultivated for 48 hours in normal plasma and spleen extract. Note growth of cells filling in defects caused by scarification. Eosin and methylene blue. × 225 and × 1200.

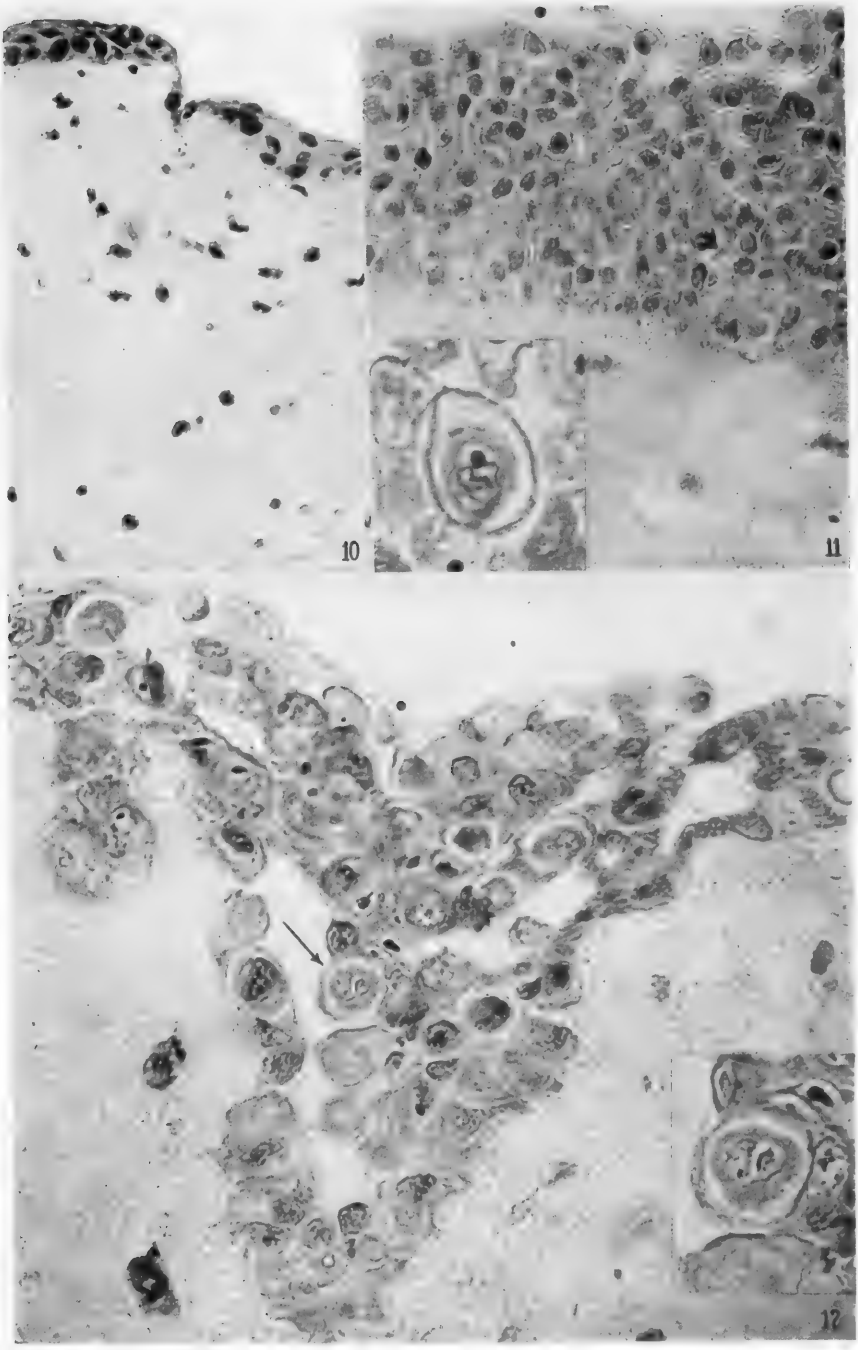
FIG. 14. Section of a 48-hour culture of a vaccine virus infected cornea. Note the thickening of the layer of epithelial cells, many of which contain vaccine bodies. Giemsa. × 225 and × 1200.

FIG. 15. Section of a 48-hour culture of a herpes virus infected cornea. Note hummock formation, amitotic giant cells, and acidophilic nuclear inclusions. Giemsa. × 225 and × 1200.




Photographed by Louis Schmidt

(Rivers *et al.*: Intracellular changes in tissue cultures)

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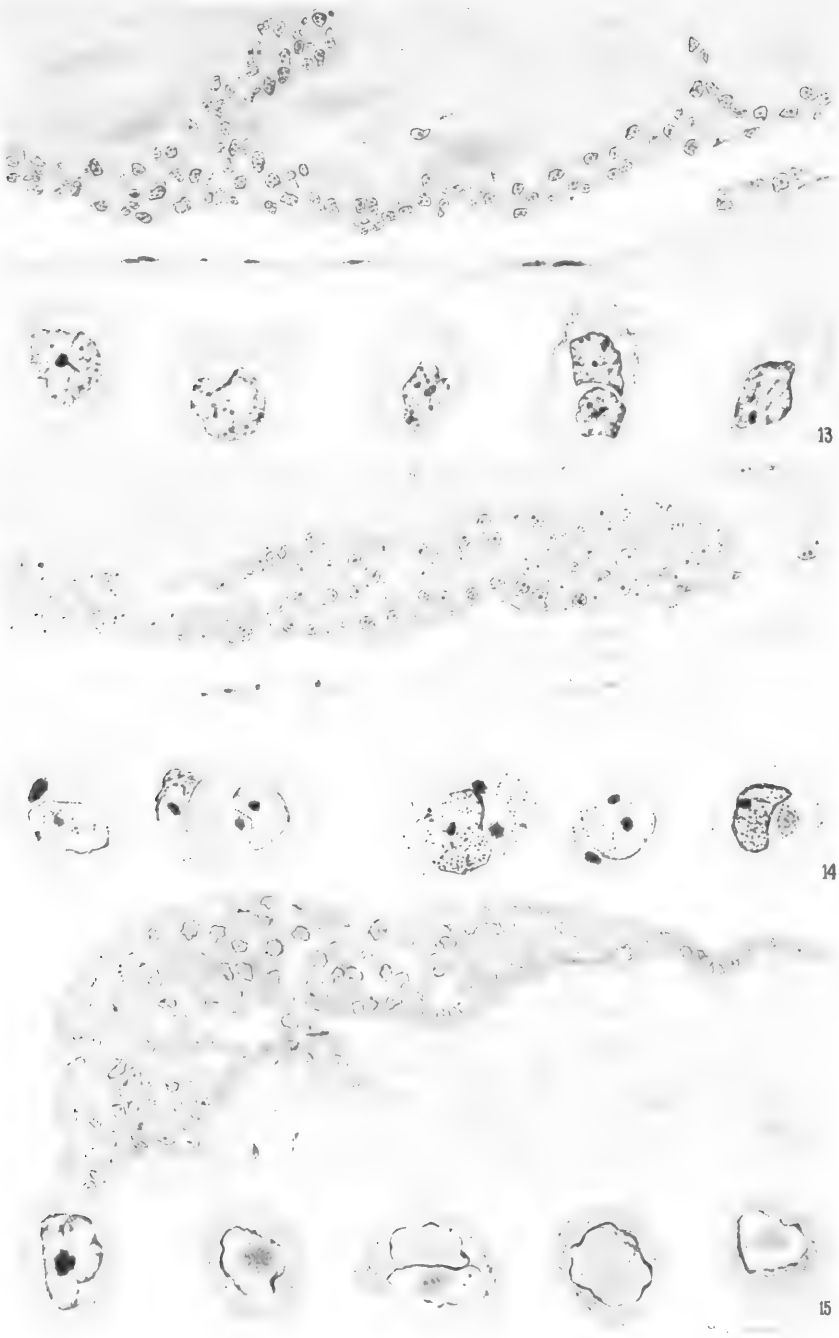
(Rivers *et al.*: Intracellular changes in tissue cultures)




(Rivers *et al.*: Intracellular changes in tissue cultures)

# A STUDY OF VACCINAL IMMUNITY IN TISSUE CULTURES

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In the preceding paper (1) a method of obtaining in tissue cultures visible evidences of infection with vaccine virus was described. The method is applicable to the study of certain phenomena related to vaccinal immunity, and the present communication deals with information secured in this manner.

For the investigation of various immunological problems, tissue cultures or tissue culture methods have been employed by Carrel and Ingebrigtsen (2), Przygode (3, 4), Foot (5), Hadda and Rosenthal (6), Lambert and Hanes (7), Bloom (8), Shwartzman (9, 10), Meyer and Loewenthal (11), Loewenthal and Micseh (12), and others. Such methods have also been used in experimental work on virus immunity. Steinhardt and Lambert (13) attempted to grow vaccine virus in cultures of normal corneal epithelium embedded in antivaccinal plasma. When these cultures were emulsified and inoculated on the skin of a rabbit, no virus was demonstrable. Later, Harde (Steinhardt) (14) reported the results of additional work. She found that cultures of normal or immune corneas in antivaccinal plasma did not support the growth or survival of vaccine virus, while preparations of immune corneas in normal plasma were less "lytic" for the virus. Recently, Nye and Parker (15) attempted to cultivate vaccine virus in normal tissues embedded in normal plasma to which serum from a hyperimmunized calf had been added. In these cultures they were unable to demonstrate virus, while in their control preparations virus was shown to be present.

Fischer's (16) results with the virus of Rous' sarcoma are at variance with those reported above by workers dealing with vaccine virus. He found that serum from immune fowls neutralized active tumor filtrates, but had no deleterious effects on sarcoma cells in tissue cultures, for, when cells from such cultures were injected into susceptible fowls, typical tumors developed. In view of these findings, he concluded that the antibodies in the immune sera were unable to injure virus bound to or situated within cells.

Todd (17) and Andrewes (18) have shown that certain antiviral sera, which inhibit the activity of viruses, are not necessarily "virucidal." From inactive mixtures or from neutral mixtures of immune serum and virus they have been able, under proper conditions, to recover active fowl plague virus and vaccine virus.

Furthermore, Andrewes (19) has reported that, if vaccine virus was injected into the skin 5 minutes before an overneutralizing dose of antivaccinal serum was administered in and around the site of inoculation, the activity of the infectious agent was not completely suppressed; whereas, if the serum was injected first, or if a mixture of virus and serum was injected, no vaccinal lesions developed. From these facts he is prepared to entertain the idea that the protective antibodies in the serum act by rendering susceptible cells insusceptible rather than by killing the virus.

Rivers and Pearce (20) found that it was possible to recover Virus III and vaccine virus from a transplantable rabbit neoplasm for at least 54 and 64 days respectively after a mixture of tumor cells and viruses had been inoculated intratesticularly. At the time when the viruses were recovered, the hosts had long since become refractory to reinoculation from without, and their sera had been "virucidal" for many weeks. Douglas, Smith, and Price (21) have obtained vaccine virus from the brains of recovered animals 41 days after inoculation. By means of cataphoresis, Olitsky and Long (22) demonstrated vaccine virus in the testicles of rabbits inoculated in the skin many months previously. Cole and Kuttner (23) were able at all times to obtain from immune animals the "salivary-gland virus" of guinea pigs, and it appears that a pig once infected continues to harbor the virus indefinitely in spite of a refractory state to reinfection from without.

The above review of the literature concerning virus immunity is not exhaustive. It is given merely to point out some of the discordant results obtained by means of tissue cultures, and to indicate a few phenomena, observed in animals infected with viruses, that are difficult or impossible of analysis in a living host. The experiments described below, in which the visible evidences of vaccinal infection in tissue cultures, inclusion bodies, that is to say, guided or aided the workers, show how certain problems in the virus field may be attacked.

## *Methods and Materials*

*Virus.*—Levaditi's neurovaccine virus prepared in the testicles of rabbits was employed in all experiments. It was found advisable to use a freshly prepared virus emulsion to which no preservative had been added.

*Tissues.*—Rabbit corneas prepared and handled as described in the preceding paper (1) constituted the tissues investigated. Normal corneas were obtained from stock animals. Immune corneas were secured from rabbits that had recovered from severe vaccinal infections in the skin or testicles. Neutralizing properties in the blood and a refractory state in the skin develop rapidly after a dermal infection with vaccine virus. An immune state of the cornea, however, develops very slowly under such conditions, and at times a completely refractory

condition requires several months for its appearance. In this work, no cornea was considered immune until it had been tested *in vivo* with an extremely potent virus and found refractory, or until it had recovered from the vaccinal keratoconjunctivitis induced by the test inoculation. Furthermore, it is not expedient to test one eye and then use the other, because both eyes do not always simultaneously attain an absolutely refractory state.

*Plasma and Serum.*—For each experiment fresh normal plasma or serum was collected from stock rabbits. Immune plasma or serum was obtained from rabbits that had recovered, 2 to 4 weeks previously, from severe vaccinal infections. The amount of immune plasma or serum used at any time in the experiments was known to be more than sufficient for the neutralization of the virus employed.

*Tissue Extract.*—Tissue extract was always secured from the spleens of rabbits that had supplied the normal corneas and normal plasma.

*Cultures.*—The methods of inoculating the tissues, and of preparing, examining, and testing the cultures were similar to those described in the preceding paper (1).

## EXPERIMENTAL

Numerous experiments have been performed, in which various combinations of normal and immune corneas with normal and immune sera, or with normal and immune plasmas, were tested for their reactions to, or effects on vaccine virus. For instance, bits of normal corneal tissue were immersed in immune serum for 2 hours at 37°C., washed in Ringer's solution, placed in an emulsion containing vaccine virus for 2 hours, again washed in Ringer's solution, and finally cultivated in normal plasma and spleen extract. The reverse experiment, in which the bits of corneal tissue were first placed in contact with vaccine virus and then with immune serum, was also made. At times the tissues were allowed to remain in contact with immune serum or vaccine virus overnight in the icebox. The results of such experiments have been irregular, and, inasmuch as no definite conclusions can be derived from them, a report concerning them will not be made at this time.

Certain experiments of other sorts, properly performed and controlled, yielded constant results. They were the ones in which small pieces of normal corneas, inoculated with vaccine virus either *in vivo* or *in vitro*, were cultivated in normal and in antivaccinal plasma for 24 and 48 hours, or in which immune corneas were removed from animals, washed for 2 hours in Ringer's or Tyrode's solution, placed

in a virus emulsion for 2 or 3 hours, and finally cultivated for 24 and 48 hours in normal and in immune plasma. It seems unnecessary to give in detail all the experiments. Some of the typical ones, with their results, will be described however.

### *Development of Vaccinal Lesions in Bits of Normal Corneas Cultivated in Antivaccinal Plasma*

In the study of vaccinal lesions that developed in normal corneas cultivated in antivaccinal plasma, 5 experiments, in which the corneas were inoculated *in vivo*, and 7, in which they were infected *in vitro*, were performed. Of the former, in one of which glycerolated virus was used, all resulted successfully. Of the latter, 5 resulted successfully; in one the infection was so mild that no conclusions could be drawn, and in another the cultures were sufficiently contaminated with bacteria to be rendered valueless. 2 experiments in which the corneas were inoculated *in vivo*, and 1 in which they were infected *in vitro* will be described in detail.

### *The Development in Normal Corneas Infected in Vivo and Cultivated in Normal and in Immune Plasma*

*Experiment XIV.* May 14, 1929.—Freshly prepared Levaditi neurovaccine was used. Immune plasma, known to possess sufficient neutralizing power, was collected. Both eyes of 2 normal rabbits were thoroughly cocainized, scarified, and inoculated with vaccine virus. 2 hours later normal plasma was obtained from the animals. Then the rabbits were sacrificed, the eyes were enucleated, and finally the spleens were removed for tissue extract. The eyes were washed in Ringer's solution in order to remove as many contaminating bacteria as was possible. The 4 corneas were removed and divided into 20 pieces, 4 of which were immediately placed as controls in Zenker's fluid. The remaining 16 were treated in the following manner:

A.—In clots of normal plasma and spleen extract, 4 pieces of cornea were cultivated at 37°C. for 24 hours, after which 2 were fixed in Zenker's fluid and examined for vaccinal lesions, and 2 were carefully removed from the plasma medium, gently washed in Ringer's solution, ground in a mortar, and injected into the skin of a normal rabbit as a test for the presence of active vaccine virus.

B.—4 pieces were treated in a manner similar to that employed under A with the exception that immune plasma was used instead of normal.

C and D.—Duplicates of A and B respectively, with the exception that the cultures were incubated 48 hours instead of 24.

The results of the above experiment are summarized in Table I and clearly show that normal corneas, inoculated with vaccine virus *in vivo* (2 hours) and then cultivated *in vitro* in antivaccinal plasma, developed typical lesions with Guarnieri bodies in the presence of antibodies in the immune plasma. Furthermore, when bits of normal corneas, inoculated and cultivated in a similar manner, were removed from the immune plasma, washed in Ringer's solution,

TABLE I

*Summary of Experiments XIV, XVI, and XVII*

| Exp. | Cultures | 24 hours | | | 48 hours | | |
|---|---|---|---|---|---|---|---|
| | | Sections | Rabbit inoculations | | Sections | Rabbit inoculations | |
| | | | Washed | Unwashed | | Washed | Unwashed |
| XIV | N.C. + N.P. | +++ | ++++ | 0 | ++++ | ++++ | 0 |
| | N.C. + I.P. | ++ | ++++ | 0 | +++ | +++ | 0 |
| XVI | N.C. + N.P. | ? | 0 | − | + | 0 | +++ |
| | N.C. + I.P. | ? | − | − | + | ++ | ? |
| XVII | N.C. + N.P. | ++ | 0 | +++ | +++ | 0 | +++ |
| | N.C. + I.P. | +++ | + | ± | ++ | + | + |

N = normal; C = cornea; I = immune; P = plasma; O indicates that test was not made; ? indicates doubtful lesions or doubt regarding presence of active virus; − indicates absence of lesions or absence of active virus; + s indicate the severity of the lesions with Guarnieri bodies as seen in sections of the bits of cornea, or the amount of active virus as determined by the size of the lesions at the sites of inoculation of the bits of cornea in the skin of rabbits.

emulsified, and injected into the skin of a normal rabbit, an abundance of active vaccine virus was demonstrated. The lesions in the bits of cornea cultivated in immune plasma were not as extensive as were those in the pieces of tissue set up in normal plasma. Nevertheless, the lesions were definite and frequently involved the new cells growing in to repair the defects caused by scarification. Inasmuch as these young cells were formed after the bits of cornea were placed in immune plasma,* the indications are that new cells arising from old infected

* This is indicated by the fact that in the controls, promptly fixed in Zenker's fluid after the inoculated eyes were enucleated, no growth of cells had taken place and no Guarnieri bodies were present.

cells or in the immediate vicinity of involved cells are capable of becoming infected even when the tissues are growing in a medium of immune plasma.

*Experiment XVI.* June 4, 1929.—The inoculations, collections of normal and immune plasmas, and preparations of cultures, were similar to those described in Exp. XIV. The following tests and controls, subsequently handled as indicated, were set up.

1 bit of inoculated normal cornea in normal plasma and spleen extract incubated 24 hours; examined histologically.

1 bit of inoculated normal cornea in normal plasma and spleen extract incubated 24 hours: ground up with at least 1 cc. of the plasma and injected into the skin of a rabbit.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 2 hours; examined histologically.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 24 hours; removed from the plasma clots, washed in Ringer's solution, emulsified, and injected into the skin of a normal rabbit.

2 bits of inoculated normal cornea in immune plasma and spleen extract incubated 24 hours; ground up with at least 1 cc. of the plasma and injected into the skin of a rabbit.

1 tube of immune plasma and spleen extract incubated at 37°C. for 24 hours. Then the plasma was tested for its neutralizing power.

1 tube of immune plasma and spleen extract placed in the icebox for 24 hours. Then the plasma was tested for its neutralizing power.

Duplicates of the above preparations were set up and incubated at 37°C. or stored on ice for 48 hours instead of 24.

The results of Experiment XVI are shown in Table I. The control portions of immune plasma held in the icebox and in the incubator for 24 and 48 hours were found to be more than capable of neutralizing the amount of virus in the bits of cornea at the beginning of the experiment. It seemed, however, that both of the 48-hour specimens had lost some of their neutralizing properties. This phenomenon is undergoing further investigation. There was very little evidence of vaccine virus either in the sections of the 24-hour cultures or in the rabbits inoculated with emulsions of the cultures. This was equally true of preparations of normal cornea in normal or in immune plasma. The demonstration of virus in the 48-hour cultures of normal cornea in immune plasma was accomplished through the removal, by gentle washing, of the immune plasma from the bits of tissue before they were

emulsified for test inoculations in rabbits. The failure to separate the infected tissues from the immune plasma may account for the fact that other workers have been unable to demonstrate active virus in cultures of normal tissues infected and then grown in immune plasma. At times,—this fact is well exemplified by the next experiment,—it was possible to demonstrate virus in such cultures even when the pieces of cornea had not been entirely freed from the antivaccinal plasma.

### *The Development in Normal Corneas Infected in Vitro and Cultivated in Normal and in Immune Plasma*

*Experiment XVII.* June 11, 1929.—This experiment was a repetition of Experiment XVI with 2 exceptions, viz., the corneas were inoculated *in vitro* instead of *in vivo*, and 2 pieces of the tissue were chosen for controls after they had been inoculated with vaccine virus and before the cultures were set up. 1 of the control bits of cornea was ground up with 0.5 cc. of normal plasma, the other with 0.5 cc. of immune plasma. These emulsions, tested in the skin of a normal rabbit, showed that the immune plasma neutralized the virus in the freshly inoculated cornea while the normal plasma did not. Thus it was demonstrated that 3 cc. of immune plasma, the amount used in the cultures, was at least 6 times greater than the amount necessary to neutralize the virus in the bits of cornea at the time when the cultures were set up.

From the results of Experiment XVII, which are summarized in Table I, it is evident that normal corneas infected *in vitro* and cultivated in antivaccinal plasma developed typical vaccinal lesions with Guarnieri bodies and that such cultures contained active vaccine virus. There was little or no difference in the size of the lesions produced by the washed and unwashed bits of cornea from the 48-hour cultures. A difference was apparent, however, in the tests made with the tissue from the 24-hour cultures.

### *Reaction of Immune Corneas to Vaccine Virus*

In the investigations concerning the nature of the *in vitro* reaction of immune corneas to vaccine virus, 6 experiments were performed. 2 of these must be disregarded, inasmuch as the corneas were not tested *in vivo* for immunity before being used in the cultures. For immunity to vaccine virus is relative, and certain tissues more slowly

attain a completely refractory state than do others. This is particularly true of corneal epithelium. In fact, one cornea of an animal may be refractory while the other is slightly susceptible. Moreover, the results of skin tests are not reliable indicators of the state of corneal immunity. Therefore, in order to be correctly informed concerning the state of immunity of any cornea, one must test it directly with a freshly prepared potent virus. In 3 of the 4 experiments which were properly controlled, the immune corneas exhibited no typical vaccinal lesions, while in 1 the corneas showed evidences of a very mild infection associated with a few Guarnieri bodies. 2 of the experiments will be described in detail.

### *Immune Corneas Infected in Vitro and Cultivated in Normal and in Immune Plasma*

*Experiment XI.* Jan. 18, 1929.—A normal rabbit received multiple intradermal inoculations of vaccine virus. Feb. 8, 1929, the animal was reinoculated in the skin and corneas. A mild reaction occurred in the skin, and a moderately severe one in the eyes. April 16, 1929, the rabbit was sacrificed for immune plasma and immune corneas. A normal animal was likewise sacrificed for plasma, corneas, and spleen extract. A fresh testicular virus emulsion was prepared. The immune and normal corneas were inoculated with vaccine virus *in vitro* (3 hours) in the usual manner; cultures were prepared and incubated at 37°C. for 48 hours; the tissues were than fixed and examined with the following results.

1.—Normal corneas infected and cultivated in normal plasma revealed severe vaccinal lesions with numerous Guarnieri bodies.

2.—Immune corneas infected and cultivated in normal plasma evidenced a very mild infection associated with a moderate number of vaccine bodies.

3.—Immune corneas infected and cultivated in immune plasma showed an exceedingly mild infection with only an occasional Guarnieri body.

In the above experiment, although evidences of a mild infection were found in the immune corneas, a definite difference in the extent of the involvement of the normal and immune tissues was evident. The experiment was repeated with an animal that had been immunized and tested in a manner similar to that used upon the immune animal above, and no evidences of a vaccinal infection were found in the immune corneas, while moderately severe lesions were noted in the normal corneas. In these experiments, no tests for the presence of

active vaccine virus in the cultures were made. Such tests, however were conducted on 2 other occasions, and the results, one set of which will be described below, were identical in both instances.

*Experiment XVIII.* Three rabbits received multiple intradermal inoculations of vaccine virus, Jan. 24, Feb. 4, and Feb. 18, 1929, respectively. June 3, 1929, the animals were reinoculated in the skin and corneas. All were refractory to reinfection of the skin. The first animal, however, had a mild "take" in the right eye, none in the left; the second evidenced a mild infection in both eyes; the third showed a reaction in the right eye, none in the left. The lesions in the eyes rapidly healed, and the corneas were used as immune tissues. The corneas that had evidenced "takes" and had recovered were labeled A, while those that had shown no reaction were marked B. A s and B s were run in parallel series, but, inasmuch as the results in the 2 series were identical, they will not be described separately.

June 18, 1929, the 3 immune rabbits were sacrificed, and the corneas were removed, scarified, divided into 4 pieces each, and washed for 2 hours in Tyrode's solution at 37°C. Then, the bits of cornea were immersed for 2 hours in a fresh virus emulsion at 37°C. Immune plasma was collected from rabbits recently (2 to 4 weeks) recovered from severe vaccinal infections in the skin. Normal plasma, normal corneas, and spleen extract were obtained from stock rabbits. The normal corneas were scarified and infected in a manner similar to that employed for the immune tissues. Either 4 or 8 tubes of each combination of the materials enumerated above were prepared. One half of the preparations was incubated 24 hours, while the remaining half was held at 37°C. for 48 hours. At the end of each period of incubation the preparations were again divided; the bits of cornea in one half were fixed and stained for histological examination, while those in the other half were carefully removed from their plasma clots, washed in Ringer's solution, emulsified, and injected into the skin of normal rabbits. Lesions produced by these intradermal inoculations were excised, emulsified, and rubbed on the scarified skin of another set of normal rabbits in order to demonstrate typical vaccinal pustules. Control specimens of immune plasma held on ice or in the incubator for 24 and 48 hours were tested for neutralizing properties. The results of the experiment are given in detail below.

### *24-hour Preparations*

1.—Normal cornea in normal plasma: Mild vaccinal lesions found, virus present.

2.—Immune cornea in normal plasma: No vaccinal lesions observed, virus present.

3.—Immune cornea in immune plasma: No vaccinal lesions observed. Virus not demonstrable.

4.—Specimens of immune plasma held on ice and in the incubator appeared to have retained full neutralizing power.

*48-hour Preparations*

5.—Normal cornea in normal plasma: Pronounced vaccinal lesions observed, large amount of virus present.

6.—Immune cornea in normal plasma: No vaccinal lesions found, virus present but less in amount than that in tissue of 5.

7.—Immune cornea in immune plasma: No vaccinal lesions seen, virus present but less in amount than that found in the cultures of 6.

8.—Specimens of immune plasma held on ice and in the incubator seemed to have lost some of their neutralizing power. They were still capable, however, of neutralizing an amount of virus greater than that taken up by the bits of cornea during the process of inoculation.

From the results of Experiment XVIII it appears that the epithelium of normal and immune corneas did not react similarly to vaccine virus, as evidenced by the fact that typical vaccinal lesions with Guarnieri bodies were observed only in the former. Virus, however, was demonstrated in the preparations of normal corneas in normal plasma, in the 24-hour cultures of immune corneas in normal plasma, and in the 48-hour cultures of immune corneas in normal and in immune plasma. Furthermore, the amount of virus in the 48-hour preparations of immune corneas seemed, as determined by the size of lesions produced in rabbits, to be greater than that in the 24-hour cultures. At present, it is impossible to state whether this apparent increase in the amount of virus was due to an actual multiplication of the infectious agent. Nevertheless, the striking fact remains that vaccine virus can at least survive for 48 hours in cultures of immune corneas in normal plasma and to a less extent in preparations of immune corneas in antivaccinal plasma.

## DISCUSSION

It should be noted that the *in vitro* vaccinal infection of normal corneal epithelial cells and the *in vitro* refractory state exhibited by immune corneal epithelium occurred without the direct intervention of such structures as cells of the reticulo-endothelial system and white blood cells. What part, if any, the various tissues of the body played in bringing about the refractory state of the immune corneas before they were removed from the body is not known.

Adequate explanations of some of the observations reported in this

paper must await further experimental work. Interpretations for a few of the results, however, are at hand, but even these are associated with a certain amount of speculation. The fact that vaccinal lesions with Guarnieri bodies developed in normal corneas inoculated *in vitro* and then cultivated in antivaccinal plasma does not appear unusual, provided one assumes that the activity of virus situated intracellularly could not be influenced by the antibodies in the immune plasma. This assumption at least agrees with the experimental observations of Rous and Jones (24) who found that intracellularly situated typhoid bacilli and red blood cells were not susceptible to such injurious agents as potassium cyanide and antisera. The results of the experiments in which immune corneas* were cultivated in normal and in immune plasma appear to support the idea of Todd (17) and Andrewes (18) that substances protective against viruses are not necessarily virucidal and also to fall in line with the observations recorded concerning the persistence of viruses in immune animals (20, 21, 22, 23).

SUMMARY

Normal corneas inoculated *in vitro* with vaccine virus and then cultivated in antivaccinal plasma developed typical vaccinal lesions associated with Guarnieri bodies. In such cultures, after an incubation period of 24 or 48 hours, active vaccine virus was demonstrated by means of appropriate methods.

Immune corneas inoculated *in vitro* with vaccine virus and then cultivated in normal or in antivaccinal plasma revealed either very mild vaccinal lesions or none at all. In some of the cultures after 24 and 48 hours of incubation, active vaccine virus was demonstrated.

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* Results reported in this paper are different in certain respects from those secured by Dr. Andrewes (personal communication to Dr. Rivers). Such differences, no doubt, may be accounted for by the fact that he conducted his experiments with Virus III in testicular tissue cultures made according to Maitland's technique.

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# CUTANEOUS REACTIONS TO THE POLYSACCHARIDES AND PROTEINS OF PNEUMOCOCCUS IN LOBAR PNEUMONIA

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PLATE 32

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Clough, 1915, (1) employing a solution of alcohol precipitable protein derived from pneumococci, tested patients suffering from pneumonia by intradermal injections. There resulted in some instances a slightly elevated discrete papule surrounded by an indefinite zone of hyperemia; all of the reactions faded in 24 hours. Little or no difference was noted between the reactivity of patients with pneumonia and normal individuals, nor was the reaction affected by the stage of the disease in which the test was made. Weil, 1916, (2) prepared material from cultures of Type I pneumococcus. He allowed the organisms to autolyze at 37°C. for 2 hours, followed by heating at 60°C. for 1 hour. 0.1 cc. to 0.2 cc. of the supernatant fluid of this solution was used for skin tests. He obtained no reaction in patients tested during the acute phase of the disease. However, 1 to 21 days after crisis, injections produced a circumscribed erythema with slight infiltration at the point of puncture. A few cases did not react at any time; normal persons and patients suffering from other diseases gave no uniform response. In 1917, Steinfeld and Kolmer (3) carried out a series of skin tests, employing heat killed Type I, II, and III pneumococci. The reactions were read 48 hours after the injection; when positive the local lesion lasted 4 to 5 days. Six of 19 pneumonia patients gave positive reactions with one or another of the test organisms. No relation was noted between the type of pneumococcus eliciting the skin response and the causative organism of the disease. All of the positive reactions were obtained in patients tested after the crisis. Tests on control individuals were recorded as being negative. Weiss and Kolmer, 1918, (4) employed in a study of the cutaneous reaction of pneumonia patients, so-called pneumotoxin prepared by dissolving washed pneumococci in 2 per cent sodium choleate. This material was freshly made and standardized in terms of minimal lethal doses for guinea pigs. The reactions were read 48 hours after the injection. In adults positive reactions were obtained in 100 per cent of 31 acute cases. The period of reactivity ranged from the fifth day (2 days before crisis) to the thirteenth day (6 days after crisis) of disease. In children, tests were positive during the acute phase of illness and negative after recovery. No correlation was noted between a

positive test and the type of pneumococcus derived from the sputum. Bigelow, 1922, (5) prepared several varieties of test materials; they consisted of heat killed pneumococci, autolysates, and solutions. In some instances the material was derived from pooled cultures of Types I, II, III, and Group IV pneumococci; other preparations were prepared from organisms of a single type. In tests on 104 patients, he described two types of reaction. One of these was designated as type specific. It was described as an indurated papule surrounded by an areola, which reached its height 28 to 32 hours after inoculation. This form of response was best obtained with autolysates. It was considered by the author to be type specific since in 8 of 11 cases the material eliciting a positive reaction corresponded in type to that of the pneumococcus recovered from the patient. In 3 patients this correlation did not occur. The second or "common" reaction was obtained with most of the preparations. Lesions of this character reached their height in 18 hours and faded in 24 to 36 hours. It was obtained in 42.3 per cent of those tested and, in most instances, was elicited after crisis had taken place. Forty-five per cent of control individuals also gave this reaction. The author concludes that positive tests occur most commonly at the time during which antibodies would be present in greatest concentration. Herrold and Traut, 1927, (6) employed filtrates derived from a 5 day culture of an avirulent Type I pneumococcus. Reactions, when positive, appeared in about 12 hours and reached the maximum after 18 hours. Readings were made at the end of 24 hours. Of 38 patients acutely ill with pneumonia, 73 per cent failed to react, while only 15 per cent of normal persons, gave negative tests. Eight patients were repeatedly tested; one of these, negative at first, later became positive; another, originally positive, subsequently became negative; 2 were positive and 4 negative throughout the period of observation. They state that they found no relation between the material used for positive tests and the type of pneumococcus causing the infection.

From this review of the literature it will be seen that uniform results have not been obtained in pneumonia patients tested intracutaneously with pneumococcus products. It will be further noted that, with the exception of Clough (1) who used pneumococcus protein, the tests have been made with autolysates, heat killed organisms, solutions of pneumococci, and culture filtrates. Materials of this character are known to contain a variety of bacterial products, such as protein, carbohydrate, pneumotoxin, hemolysin, and the purpura producing substance, as well as other derivatives of enzyme action, in varying concentrations. Attention is directed to this point since the results reported in this paper demonstrate that the composition of the material used for injection influences the character of the reaction.

In the experiments presented in the present communication measured quantities of two distinct constituents of the pneumococcus cell

were separately employed for intradermal injection into patients acutely ill with and convalescent from lobar pneumonia. The material used for testing consisted of: 1. The purified, protein-free, carbohydrates of Types I, II, and III pneumococci,—the so-called soluble specific substances; 2. The somatic proteins of Pneumococcus. This material, the acetic acid precipitable fraction, consists largely of nucleo-protein. A description of both carbohydrate and protein is given further on. It has been shown by Avery and Heidelberger (7) that the pneumococcus carbohydrates are the type specific components; they react only in homologous type specific anti-sera, and, in purified form, are not antigenic. The protein fraction, on the other hand, is a common constituent of the pneumococcus species; antibodies elicited by immunization with it, react with protein derived from any strain of this organism.

In addition to the intradermal injection of the polysaccharides and protein, the experiments were supplemented by obtaining serum from the patients just prior to the skin test, and titrating for the presence or absence of antibodies reactive with the test solutions.

## *Material and Methods*

*Skin Testing Materials. 1. Soluble Specific Substances.*—Type specific polysaccharides from each of three pneumococcus Types (I, II, and III) were employed. They were obtained in purified state according to the method employed in this laboratory by Heidelberger and Avery (8). As demonstrated by Heidelberger, Goebel, and Avery (9), these substances possess the chemical properties of complex sugars; they contain no phosphorous, no sulfur, and give none of the usual protein color tests. Type II and Type III substances are nitrogen-free. The Type I substance differs from the other two in containing nitrogen as an apparently essential component. The total nitrogen present in Type I is 5 per cent, half of which is present in the amino form. Despite the presence of nitrogen, the substance fails to give any of the protein color reactions. For skin tests, the polysaccharides were dissolved in *freshly* prepared physiological salt solution in such concentration that 0.1 cc. of solution contained 0.01 mgm. of specific polysaccharide. For purposes of sterility the solutions were heated at 100°C. for 10 minutes before being used.

*2. Pneumococcus Protein.*—The material was prepared according to the method described by Avery and Morgan (10). Cultures of an R strain of pneumococcus originally derived from Type II S organisms served as the source from which the protein was obtained. The preparations were filtered through Berkefeld filters and tested for sterility before being used for injection. The material was stand-

ardized by nitrogen determinations and diluted in fresh physiological salt solution so that 0.1 cc. of solution contained 0.01 mgm. of protein.

In each instance the material was injected in 0.1 cc. amounts into the skin on the flexor surface of the forearm.

*Titration of Sera for Antibodies.*—Blood, obtained from patients by venapuncture, was allowed to clot, centrifuged, and the clear serum pipetted off. Tests for precipitins reactive with carbohydrate and protein were separately carried out. 0.2 cc. serum diluted with 0.3 cc. physiological salt solution was mixed with 0.5 cc. of varying dilutions of the precipitinogens. Readings were made after the tubes had been allowed to incubate at 37°C. for 2 hours and then placed in the ice-box over night. It was found that type specific antibodies were more easily demonstrable by agglutination tests with the intact type specific cells. Consequently this test was more commonly used. 0.5 cc. of varying dilutions of serum was mixed with 0.5 cc. of heat killed organisms of each of Types I, II and III. The tubes were incubated at 37°C. for 2 hours and kept on ice over night. A positive reaction was detected by the presence of the characteristic disc formation.

On admission to the hospital patients were injected intradermally with Types I, II and III carbohydrate solutions, protein solution, and a control injection of fresh physiological salt solution. The tests were often repeated several times both during the acute phase of the illness and after crisis. Freshly prepared materials were always employed for injection. This precaution was considered to be of considerable importance since it was found that solutions which had been standing for several days often gave rise to immediate reactions which were deceptive. In addition to skin tests, serum obtained, in most instances at the time of intradermal injection, was titred for antibodies reactive with the carbohydrates and protein. Typing of the pneumococcus derived from the sputum of the tested patients was also carried out.

### *Cutaneous Reactions to Specific Polysaccharides*

Observation on the skin reaction of nineteen individuals suffering from lobar pneumonia have been made following injection of 0.01 mgm. of each of the type specific polysaccharides. Classified on the basis of the pneumococcus type derived from the patient, the cases may be divided into: 11 cases of Type I infection; 3 of Type II; 2 of Atypical Type II; and 3 of Group IV. Unfortunately, no instances of Type III pneumococcus infection were available during the period of this investigation.*

* While this communication was in press a patient, convalescent from Type III pneumococcus pneumonia, has shown a positive reaction with the Type III polysaccharide. The patient's serum contained type-specific antibodies at the time of the positive test.

In the 11 cases of Type I infection, 10 reacted to Type I S carbohydrate and none to the Type II or III substances. Of the 3 Type II cases one reacted to Type II polysaccharide, whereas all three were negative to the sugars derived from Types I and III. The 2 atypical Type II and the 3 Group IV cases were entirely negative.

With regard to the time of appearance of a positive reaction, none of the cases before recovery reacted positively. Of the 11 cases in which a positive test was obtained, 10 reacted at the time of recovery. One patient, suffering from Type I pneumococcus pneumonia did not give a positive skin test until the twenty-sixth day after recovery from the acute phase of the illness. He had persistent fever, and evidence of pleurisy during this time and only after these signs of persistent infection began to subside did the skin test become positive.

A positive reaction, when obtained, was very striking and may be described as follows:

> Ten to twenty minutes after the intradermal inoculation, there appears at the site of injection a wheal-like swelling with intense white edema. Surrounding the wheal, a zone of erythema appears which becomes increasingly larger and more intense. The edges are irregular due to the "pseudopods" of erythema extending in different directions. The height of the reaction occurs between 30 and 60 minutes after the inoculation. (See Figs. 1 and 2.) Within another hour the acute phase has entirely faded; a gradual regression then takes place leaving a firm, pale, edematous area which may require 24 hours or longer to disappear completely. Reactions of this character have been found to be strictly type specific and to occur only with the polysaccharide corresponding to the serological type of the infecting organism. At the site of injection of the carbohydrates of heterologous types and of the salt solution, no reaction appeared. The "wheal and erythema" form of reaction, just described, differs strikingly from the delayed response elicited by the bacterial protein.

In testing the sera of patients, it was found that in all instances in which a positive skin reaction was elicited with the specific carbohydrates, agglutinins for the homologous type of pneumococcus and precipitins for the reacting polysaccharide were present. Furthermore, the time at which specific antibodies were demonstrable in the circulation has been coincident with the development of a positive skin reaction. However, the fact that individuals may possess circulating specific antibodies without responding to the intradermal injection of the homologous specific substance will be subsequently shown.

Ten of the 11 cases in which a definite skin response to the homolo-

gous specific polysaccharide was obtained, suffered from Type I pneumococcus infection. Eight of these cases were treated with Type I antipneumococcus horse serum in 100 cc. doses at six hour intervals until recovery. Regardless of the amount of serum administered the skin reaction did not become positive until the patient had clinically recovered. The investigations are not as yet sufficiently extensive to determine the exact relations between specific antibodies introduced therapeutically and the occurrence of a positive skin reaction. However, the results are suggestive that the production of a local response with the specific polysaccharide may, under certain conditions, be helpful in determining when sufficient serum treatment has been given. Studies are being continued in an attempt to determine this point.

The single instance of Type I infection, which failed to react to the Type I carbohydrate occurred in a patient who developed pneumococcus meningitis and died on the twentieth day of disease. This patient, after receiving 800 cc. of serum, possessed demonstrable circulating Type I antibodies, but gave only doubtful or negative skin test. Another individual, after recovering from the acute phase of the illness, continued to run a low grade fever and showed signs of pleurisy. He reacted positively to the Type I specific substance only after beginning cessation of infection. These two cases suggest that persistence of infection, even though specific antibodies are present, may inhibit the skin response.

In two patients suffering from Type I pneumococcus pneumonia, no serum was administered. In both instances a positive skin response was obtained after recovery, indicating that a positive test is not dependent upon the presence of therapeutic serum. Of three Type II cases included in this series one gave a typical positive reaction to the Type II specific polysaccharide. The reaction first appeared as the result of a test made the day after crisis; the patient's serum at this time contained Type II agglutinins and precipitins. The other two Type II patients failed to react even to 0.1 mgm. of specific substance. These failures occurred despite the fact that the blood serum, in each case, contained specific Type II antibodies and that all evidence of infection had completely subsided. As formerly noted the two atypical Type II and 3 Group IV cases of pneumococcus in-

fection gave no response at any time to the skin tests with the polysaccharides of Types I, II, or III.

In all patients, tests were repeated every few days during convalescence. Those patients in whom the reactions were positive on several occasions and subsequently became negative, ceased to react at the time of disappearance of specific antibodies from the circulation.

In Table I a protocol of five cases is given, which serves to show the time of appearance and duration of skin sensitivity in relation to both recovery of patient and occurrence of demonstrable specific antibodies in the circulation.

Cases Ka. and La. suffered from Type I pneumococcus infection and were treated with Type I antipneumococcus horse serum. Case Ka. is interesting in that skin sensitivity to Type I carbohydrate and circulating Type I antibodies have persisted for 127 days after recovery; observations on this patient are being continued. Case La. first reacted on the day after recovery; three days later his capacity to react had ceased coincident with the disappearance of specific agglutinins and precipitins. Patient Ca., the case of pneumococcus meningitis referred to above failed to give a definite positive reaction at any time. Cases St. and Mo. were instances of spontaneous recovery from Type I and Type II pneumococcus infections, respectively. They each gave typical responses after crisis and continued to do so, as long as demonstrable circulating antibodies were present. In the other patients, who gave a positive skin test, the same relations were found to hold as those presented in Table I.

The local reaction resulting from the intradermal injection of 0.01 mgm. of the type specific polysaccharides presented three distinctive characteristics; first, the carbohydrate eliciting a response was always homologous in type to that of the infecting organism; second, both the gross appearance of the lesion and the rapidity with which it developed and retrogressed were characteristic of the typical wheal and erythema type of reaction; third, the capacity to react, when evident, developed at the time of the patient's recovery, and was, in this group of cases, correlated with the presence of homologous type specific agglutinins and precipitins.

### *Cutaneous Reactions to Pneumococcus Protein*

The local reaction resulting from the intradermal injection of 0.1 mgm. pneumococcus protein presented distinct differences from that

*Skin Reactions with Pneumococcus Polysaccharides in Re*

| Day of disease..... | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ka.<br>Age 14 | Agglutinins<br>Precipitins | | | | | | −<br>− | +++<br>+ | | | | | ++<br>+ |
| Type I | Skin Test. | | | | | | neg. | pos.<br>C | | | | | pos. |
| La.<br>Age 34 | Agglutinins<br>Precipitins | | | | | | −<br>− | +<br>+ | | | −<br>− | | |
| Type I | Skin Test. | | | | | | neg. | pos.<br>C | pos. | | neg. | | |
| Ca.<br>Age 55 | Agglutinins<br>Precipitins | | | | −<br>− | ++<br>+ | | +++<br>+ | | | | | ++<br>+ |
| Type I | Skin Test. | | | | neg. | neg. | neg. | neg. | | | | | ? |
| St.<br>Age 33 | Agglutinins<br>Precipitins | | | −<br>− | | | | | | | | | |
| Type I | Skin Test. | | | neg. | | | | C | | | | pos. | |
| Mo.<br>Age 33 | Agglutinins<br>Precipitins | | | | −<br>− | | | ++<br>+ | | | | +++<br>+ | |
| Type II | Skin Test. | | | | neg. | | | pos.<br>C | | | | pos. | |

Agglutinin titre in terms of highest positive serum dilution. Agglutination characterized by disc formation.

− = Completely negative.
± = Doubtful agglutination in serum dilution of 1:2.
+ = Agglutination in serum dilution of 1:2.
++ = Agglutination in serum dilution of 1:10.
+++ = Agglutination in serum dilution of 1:20.
++++ = Agglutination in serum dilution of 1:40.

I

*re of Circulating Specific Antibodies and Recovery of Patient*

| 4 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 26 | 27 | 28 | 29 | 49 | 97 | 109 | 133 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | ++ | | | | | + | | + | + | + | + |
| | | | | | | | | + | | | | | + | | + | + | + | + |
| | | | | | | | | pos. | | | | | pos. | | pos. | pos. | pos. | pos. |
| | | | | | | | | | − | | | | | | | | | |
| | | | | | | | | | − | | | | | | | | | |
| | | | | | | | | | neg. | | | | | | | | | |
| | | | | | | Death Meningitis | | | | | | | | | | | | |
| | | | ? | | | | | | | | | | | | | | | |
| + | | | | | | | | | + | | | | | ± | | | | |
| + | | | | | | | | | ± | | | | | − | | | | |
| pos. | | | | | | | | | pos. | | | | | ? | | | | |
| +++ | | | | | ++++ | | | | | | | | | | (47) | | | |
| + | | | | | + | | | | | | | | | | − | | | |
| pos. | | | | | pos. | | | | | | | | | | neg. | | | |

Precipitin test = 0.3 cc. serum + 0.2 cc. salt solution + 0.5 cc. 1–40,000 dilution of polysaccharide.

Skin test:

pos. = positive reaction.

? = doubtful reaction.

neg. = negative reaction.

C = day of crisis.

TABLE II

*Skin Reactions to Pneumococcus Proteins in Relation to Titre of Circulating Protein Precipitins and Recovery of Patient*

Type I

| Day of disease | | Ka. age 15 | | | | | | Ga. age 17 | | | | *Co. age 26 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 | 7 | 12 | 22 | 29 | 36 | 3 | 4 | 6 | 13 | 4 | 16 | 23 | 36 |
| Protein Precipitin Titre. | 1–500 | ++ | Crisis. | + | +++ | +++ | +++ | +++ | +++ | Crisis. | +++ | ++ | ++± | ++ | +± |
| | 1–1000 | ++ | | +± | ++ | ++ | ++ | ++ | +++ | | +++ | +± | ++ | ++ | +± |
| | 1–2000 | + | | + | ++ | ++ | + | +± | +++ | | +± | + | + | ++ | + |
| | 1–4000 | ± | | − | ± | + | + | + | ++ | | ++ | ± | − | + | ± |
| | 1–8000 | − | | − | ± | ± | ± | + | ++ | | + | ± | − | − | − |
| | 1–16000 | − | | − | − | − | − | ± | + | | ± | − | − | − | − |
| Skin test. | | neg. | | ? | pos. | pos. | pos. | neg. | neg. | | pos. | neg. | neg. | neg. | neg. |

* Patient Co. had a prolonged illness followed by recovery.

Type II

| Day of disease | | Fl. age 33 | | | | | Lo. age 24 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 | 8 | 10 | 20 | 27 | 2 | 5 | *7 | 17 | 24 |
| Protein Precipitin Titre. | 1–500 | 0 | Crisis. | + | +± | ++ | ++ | ++ | ++ | +++ | +++ |
| | 1–1000 | 0 | | ± | + | + | ++ | ++ | ++ | +++ | +++ |
| | 1–2000 | 0 | | ± | + | + | +++ | +++ | +± | +++ | +++ |
| | 1–4000 | 0 | | ± | ± | ± | +++ | +++ | + | ++ | ++ |
| | 1–8000 | 0 | | − | − | − | +± | + | ± | + | ++ |
| | 1–16000 | 0 | | − | − | − | ± | + | − | ± | ± |
| Skin test. | | neg. | | neg. | pos. | pos. | neg. | 0 | ? | pos. | pos. |

* Day of crisis.

Type Atypical II — Group IV

| Day of disease | | Wa. age 24 | | | | Ne. age 42 | | | | Ha. age 36 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 8 | 17 | 27 | 7 | 10 | 14 | 24 | *4 | 12 |
| Protein Precipitin Titre. | 1–500 | +± | Crisis. | + | + | ++ | Crisis. | ++ | + | +++ | ++ |
| | 1–1000 | + | | + | + | ++ | | +± | + | +++ | ++ |
| | 1–2000 | + | | + | + | +± | | + | ± | ++ | +± |
| | 1–4000 | ± | | ± | − | + | | ± | − | + | + |
| | 1–8000 | − | | − | − | ± | | − | − | + | − |
| | 1–16000 | − | | − | | − | | − | − | − | − |
| Skin test. | | neg. | | pos. | pos. | neg. | | pos. | pos. | pos. | pos. |

* Day of crisis.

*Precipitin Tests:*

+++ = precipitate at bottom of tube with faintly cloudy supernatant fluid.
++ = slight precipitate with cloudy supernatant fluid.
+ = cloudy fluid.
± = faintly cloudy fluid.
− = completely negative.
0 = not done.

*Skin Tests:*

pos. = Typical delayed reaction greater than 1 cm. in diameter.
? = Reaction 0.5 − 1.0 cm. in diameter.
neg. = No reaction.
0 = Not done.

obtained with the carbohydrates. Of twelve patients tested while acutely ill, all failed to react. In eight individuals repeated injections were made both before and after recovery. Seven of them gave positive tests after crisis, and also with each repetition of the test during convalescence. These patients have not been retested since discharge from the hospital.

A test, when positive, developed slowly as compared with the reaction elicited by the carbohydrates. In 6 to 8 hours there appeared a small, dark red, circumscribed lesion, about 0.5 cm. in diameter. It gradually increased in size until a maximum was reached in 18 to 24 hours. Readings were recorded at this time. (See Fig. 3.) Regression which began after 36 to 48 hours, usually proceded slowly, and 3 to 4 days often were required for complete disappearance. When at its height, the lesion varied from 1 to 5 cm. in diameter; it consisted of a small central papule, dark red in color, surrounded by a bright pink erythema; the edges faded gradually into normal skin; slight tenderness was some times present and the local temperature often seemed to be increased. The protein reactions were similar in many respects to the tuberculin response. That this type of reaction, in contrast to the response induced by polysaccharides, was unrelated to the type of pneumococcus causing infection is borne out by the fact that of the 7 patients, in whom positive tests were obtained, 2 had Type I pneumonia, 2 Type II, 2 Atypical Type II, and 1 Group IV.

In determining the presence of pneumococcus protein precipitins, it was found that the sera of all the patients possessed antibodies reactive with this test material. The titre ranged from 1:2,000 to 1:16,000 in the different cases. However, in a single individual, variations in amount of precipitins occurring coincident with the progress of the disease were not sufficiently striking nor constant to be considered of significance. In Table II, the results of the protein skin tests are given for those cases in which repeated injections were made.

Table II shows the relation of the development of skin sensitivity both to the time of recovery from infection and to the titre of anti-protein antibodies with serum. From the data it appears that the protein sensitiveness develops after crisis, but, as contrasted with the

reaction to the carbohydrate, is unrelated to the quantity of precipitins present in the circulation.

The presence of antibodies to pneumococcus protein in the serum of human beings, has not, as a search of the literature reveals, been the subject of previous investigation. The possible significance of their occurrence is a part of this, as yet uncompleted, study. In addition to the cases cited above, the sera of 13 other pneumonia patients have been tested at frequent intervals after admission to the hospital until discharge. They were all found to possess in their serum protein precipitins in concentrations comparable to those recorded in Table II. The study of the anti-protein antibodies is being continued and a more complete report will be made in a later communication.

## DISCUSSION

Although the observations reported in this paper on a relatively small number of patients represent an investigation which is, as yet, incomplete, sufficiently definite results have been obtained to warrant presentation. It has been shown that pneumococcus polysaccharides, when injected intradermally into pneumonia patients after crisis, can bring about in some instances, a local reaction. The cutaneous response, when obtained, was always elicited by the carbohydrate homologous to the type of pneumococcus causing infection in the patient. A patient's capacity to react became manifest coincident with recovery from infection. Furthermore, skin sensitivity has been found to parallel closely the presence of circulating type specific antibodies. The two phenomena have been found to appear at about the same time, to persist for a similar period, and to disappear at about the same stage of convalescence. However, the mere presence of type specific antibodies in the circulation is not the only factor necessary for the excitation of a skin response. The fatal case complicated by pneumococcus meningitis possessed type specific agglutinins in high titre but at no time could a definite skin response be obtained. Further investigation on a larger number of cases is necessary before the underlying mechanism can be fully interpreted. However, undetermined as the problem at the present may be, it is an interesting fact that these bacterial sugars, *protein-free*, and belonging

to that group of immunologically specific substances known as haptens, are capable of producing a reaction in the skin of convalescents from pneumococcus pneumonia. The character of the skin response incited by the polysaccharides is unique in that it is urticarial-like in appearance and runs its course in 1 to 2 hours.

The reaction caused by pneumococcus protein, on the other hand, is similar both in appearance and evolution to that evoked by tuberculin. The protein reactions, when positive, reached the maximum intensity about 24 hours after injection and some times required 3 to 4 days to subside completely. Furthermore, a patient's capacity to react to protein has no relation to the type of pneumococcus causing the infection. Sufficient observations on the presence and quantitative variations of anti-protein antibodies have not yet been made to justify final conclusions. In the instances reported in this paper, antiprotein precipitins were found in all cases both before and after crisis. In any single individual the titre did not markedly vary during the course of disease, nor did it appear to influence skin sensitivity.

The data presented in this paper represents observations made in the course of lobar pneumonia. Similar tests on normal individuals and patients suffering from other diseases are being carried out and the results will be reported in a subsequent paper.

## CONCLUSIONS

I. a. Pneumococcus polysaccharides, when injected intradermally into patients convalescent from pneumonia, are capable of eliciting a response. The polysaccharide inducing a cutaneous reaction was found always to be homologous in type to that of the pneumococcus causing the infection.

b. The character of the reaction incited by the protein-free bacterial sugars is of the immediate wheal and erythema type.

c. A patient's capacity to react was found to be intimately associated both with recovery from infection and with the presence of type specific antibodies in the circulating blood.

II. a. The so-called nucleo-protein of pneumococcus, when injected intradermally, also causes a local cutaneous reaction in patients during convalescence from lobar pneumonia.

b. The local lesion resulting from the injection of protein is tuberculin-like in character, and differs from that evoked by the type-specific polysaccharides in gross appearance, time of development, and duration.

c. Individuals, acutely ill with and convalescent from pneumococcus pneumonia, possess in their circulating blood, precipitins reactive with pneumococcus protein. In the observations recorded, the concentration of anti-protein antibodies in the blood serum did not seem to influence the patient's capacity to react to intradermal injection of the protein.

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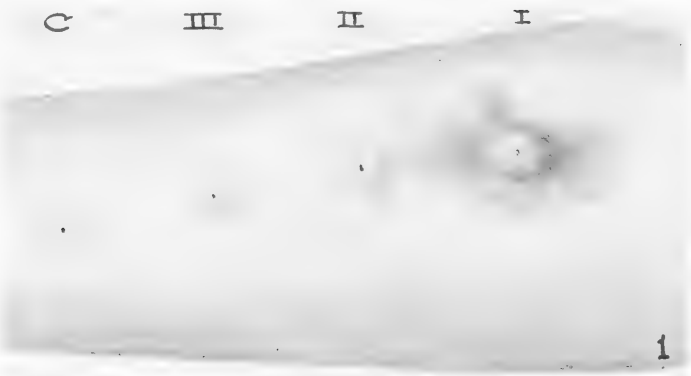
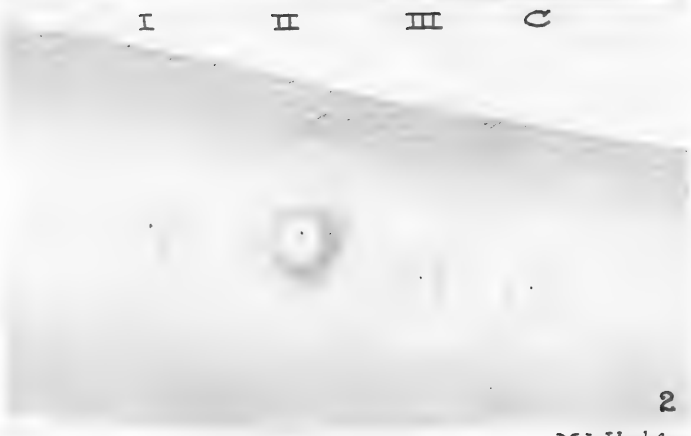
## EXPLANATION OF PLATE 32

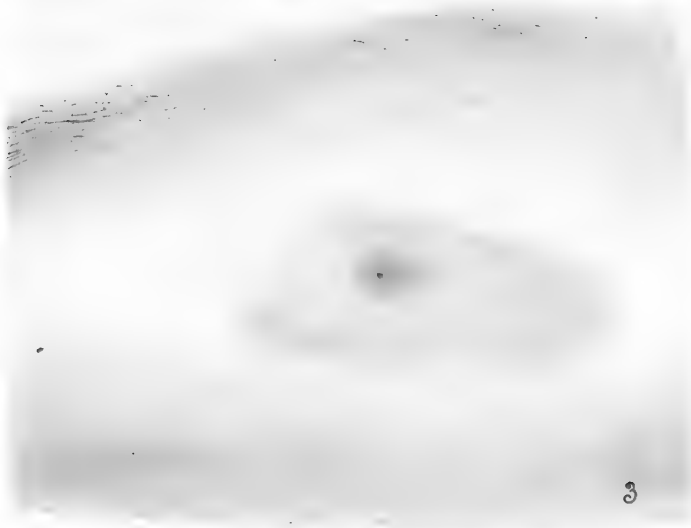
FIG. 1. Cutaneous reaction 30 minutes after the injection of 0.01 mgm. of Type I polysaccharide in a patient convalescent from Type I pneumococcus pneumonia. This illustrates the immediate wheal and erythema response to pneumococcus polysaccharide. Specific substances of Types II and III, and salt solution are negative. Roman numerals I, II, and III, designate the site of injection of Types I, II, and III carbohydrates; C represents the site of injection of salt solution. One-half natural size.

FIG. 2. Cutaneous reaction 30 minutes after the injection of 0.01 mgm. of Type II polysaccharide in a patient convalescent from Type II pneumococcus pneumonia. Specific substances of Types I and III, and salt solution are negative. Roman numerals and C serve the same purpose as in Fig. 1. One-half natural size.

FIG. 3. Cutaneous reaction 24 hours after injection produced by 0.01 mgm. of pneumococcus protein. This illustrates the delayed tuberculin-like response to pneumococcus protein. Four-fifths natural size.

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